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Roles of the Atr1-Chk1 pathway in the phytopathogenic fungus *Ustilago maydis*

PhD thesis

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Abbreviations

2D gel: Two-dimensional gel electrophoresis

9-1-1 clamp: Rad9-Rad1-Hus1 clamp

aa: amino acid

ATM: ataxia-telangiectasia mutated

ATR: ataxia-telangiectasia mutated and Rad3-related

C: DNA content

cDNA: coding DNA

CFW: calcofluor white

Ci: Curie, unit of radioactivity

CTAB: Hexadecyltrimethylammonium bromide

DAPI: 4',6-diamidino-2-phenylindole

DDR: DNA damage response

DEPC: Diethyl pyrocarbonate

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide

DSBs: Double strand breaks

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

EtBr: Ethidium bromide

EtOH: Ethanol

FACS: Fluorescence-activated cell sorting

GFP: Green fluorescent protein

Gy: gray, SI unit of absorbed radiation

HR: Homologous recombination

HU: Hydroxyurea

kb: kilobase

kDa: kiloDalton, unified atomic mass unit

MMS: Methyl methanesulfonate

MOPS: 3-(N-morpholino)propanesulfonic acid

MRN/MRX complex: Mre11-Rad50-Nbs1/Xrs2 complex

mRNA: messenger RNA

NHEJ: Non homologous end joining

NLS: Nuclear localization signal

O.D: Optical density

ORF: Open reading frame

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PEG: Plyethylene glycol

Phleo: Phleomycin or bleomycin

PIKKs: Phosphatidylinositol 3-kinase-related kinase

PMSF: Phenylmethanesulfonylfluoride

RNA: Ribonucleic acid

RNAi: RNA interference

RPA: Replication protein A

rpm: Revolutions per minute

RT-PCR: Reverse transcription polymerase chain reaction

SDS: Sodium dodecyl sulfate

SSC: Saline-sodium citrate buffer

ssDNA: Single stranded DNA

T4 PNK: T4 polynucleotide kinase

TAE: Tris base-acetic acid-EDTA

TBE: Tris base-boric acid-EDTA

TE: Tris EDTA buffer

UTAS: *Ustilago maydis* telomere associated sequences

UV: Ultraviolet

YFP: Yellow fluorescent protein

Introduction

1. Aim of the work

Dimorphism is the ability of certain fungi to switch from unicellular yeast to multicellular filamentous growth. This phenomena is extensively exploited by animal and plant pathogenic fungi, where after contact with the host a shift in the mode of growth is induced (Rooney and Klein, 2002). Changes in the environment generate a variety of signals that are perceived by the dimorphic fungi inducing a morphogenic shift as a way to adapt to the new conditions. Therefore knowing which are the signals and the events that finally lead to this morphological change would help to find new targets to avoid the infection. *Ustilago maydis* switches from a saprophytic yeast state to a pathogenic filamentous one in response to plant signals and its nuclear content. Stable filamentous growth takes place only within the maize plant showing that a signal coming from the living maize plant is essential to trigger this behavior (Nadal et al., 2008). Dimorphism, sexual development and virulence are tightly associated in the life cycles of dimorphic fungi, and in *U. maydis* all these changes are coupled to the cell cycle regulation. Activation of the virulence program leads to a cell cycle arrest and recently it was seen that a DNA damage checkpoint kinase was involved in this arrest (Mielnichuk et al., 2009). The idea of a well known kinase from the DNA damage response pathway being involved in the cell cycle arrest triggered by a developmental signal, brought up several questions. Is the cell cycle arrest a consequence of the presence of damaged DNA during the morphological changes? How does the checkpoint kinase get active?

U. maydis has been used as a model organism in the study of DNA repair processes for a long time. Holliday junctions, which are essential for the homologous recombination process and are conserved from prokaryotes to mammals, were described in *U. maydis* by Robin Holliday in the '60s. Since then, significant advances have provoked a change in *U. maydis'* consideration towards a simplified organism in which the DNA damage response and DNA repair pathways can be studied and applied to mammals. A good example of this is the *brh2* gene. *brh2* is homologous to the human BRCA2 gene and, as in mammals, it is required to maintain genome stability and proficiency in repair and recombination (Kojic et al., 2002). Other examples involve the organization of the microtubule cytoskeleton during mitosis and polar growth or the removal of the nuclear envelope in mitosis, processes that are closer to mammals rather than to budding yeast (Steinberg and Perez-Martin, 2008). In contrast to the well-

studied homologous recombination repair process, nothing is known about the DNA damage response pathway. Due to this, *U. maydis* was used to answer the questions above mentioned and to characterize the DNA damage response pathway.

2. Basidiomycetes special features

Basidiomycota is a phylum within the Fungi kingdom that includes mushrooms, bracket fungi, plant pathogens as *Ustilago maydis* and human pathogens as *Cryptococcus* species (James et al., 2006). Basidiomycetes can be distinguished from the rest of the fungi because meiosis takes place in special cells called basidia and is followed by the production of four basidiospores. Another characteristic that is shared by most of the Basidiomycetes is the presence of two phases in their life cycles: a haploid homokaryotic phase, that is usually sexually inert but can produce asexual spores and a heterokaryotic phase in which each cell contains two nuclei of different mating type. This second phase is called dikaryon and arises from the anastomosis between two haploid hyphae from different origin, is the normal prerequisite for sexual reproduction and is the predominant mycelial state in nature. The two nuclei remain discrete during somatic cell divisions thanks to the formation of clamp connections, and it's only after the formation of the fruit body that they eventually fuse in specialized reproductive cells, the basidia, where immediately undergo meiosis (Casselton, 1978; Fincham et al., 1979). Clamp connections guarantee a correct nuclear distribution that keeps the presence of two distinct nuclei per cell. Prior to cell division, a clamp cell develops on the side of the apical cell and one nucleus migrates into this clamp while the other remains in the main cell. The two nuclei divide synchronically and septa are laid down apparently across the planes of the mitotic spindles. Subsequently the clamp cell fuses with the subapical cell and its nucleus migrates into this cell (Fig. 1) (Casselton, 1978).

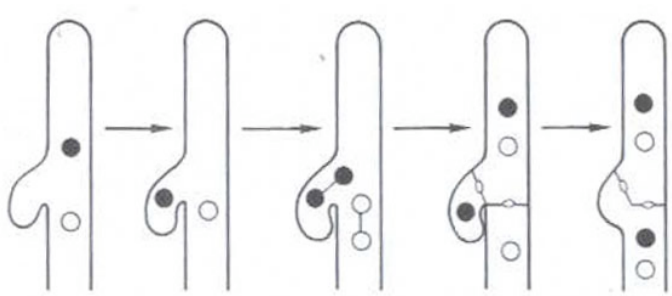


Figure 1: Dikaryon mitotic division, where the clamp cell formation and role can be observed (Casselton, 1978).

2.1. *Ustilago maydis* life cycle

Ustilago maydis is a hemibasidiomycete also known as the corn smut fungus (Christensen, 1963). Its host range is very narrow and infects only maize (*Zea mays*) and teosinte (*Zea mays* subsp. *parviglumis*) (Bolker, 2001). It is a dimorphic fungus whose life cycle is divided in two phases: a saprophytic asexual phase in which it grows in a budding yeast-like way and a pathogenic sexual phase where it grows as a dikaryon (Perkins, 1949). The change from the saprophytic phase to the pathogenic one occurs upon mating of two compatible cells, and in nature this process takes place at the plant surface (Snetselaar and Mims, 1992). *U. maydis* is a heterothallic tetrapolar species, which means that compatibility relies on two mating-type loci called *a* and *b* that have at least two alleles per locus (Raper, 1953). The *a* locus has two alleles, *a1* and *a2*, and governs cell fusion and filamentous growth through a pheromone-receptor based system (Banuett and Herskowitz, 1989; Bolker et al., 1992; Holliday, 1974b). The multiallelic *b* locus which encodes two distinct homeodomain transcription factors, *bE* and *bW*, controls the pathogenicity of the resulting dikaryon (Banuett, 1995; Puhalla, 1970). The infecting hyphae that emerges from the fusion of two conjugation tubes from compatible *a* and *b* genes forms an appressorium-like structure to penetrate the plant (Snetselaar and Mims, 1992). This appressorium is morphologically undifferentiated compared to the appressoria formed by other pathogenic fungi but is not melanized, therefore another mechanism such as production of lytic enzymes could cope with the mechanical force although this is not clear (Garcia-Pedrajas et al., 2004). Once the fungus enters the plant it grows inter and intracellularly through the epidermis and parenchyma until it reaches the vascular bundles (Snetselaar and Mims, 1994). It proliferates as a dikaryon forming a broad network of hyphae and induces the formation of tumors. Symptoms in the plant start with the appearance of chlorosis and anthocyanin streaking, which are associated with a stress response to fungal penetration from the plant, and are followed by the hyperplasia and hypertrophy of the plant tissues that will develop into tumors (Callow and Ling, 1973). Later on, inside these tumors, the hyphae become embedded in a mucilaginous matrix and nuclei fusion takes place accompanied by hyphal fragmentation. These fragments undergo several morphological changes before the formation of teliosporses (Snetselaar and Mims, 1994). Upon tumor breakage, teliospores are released into the air and their germination produce four haploid basidiospores, closing the life cycle (Ingold, 1983) (Fig. 2).

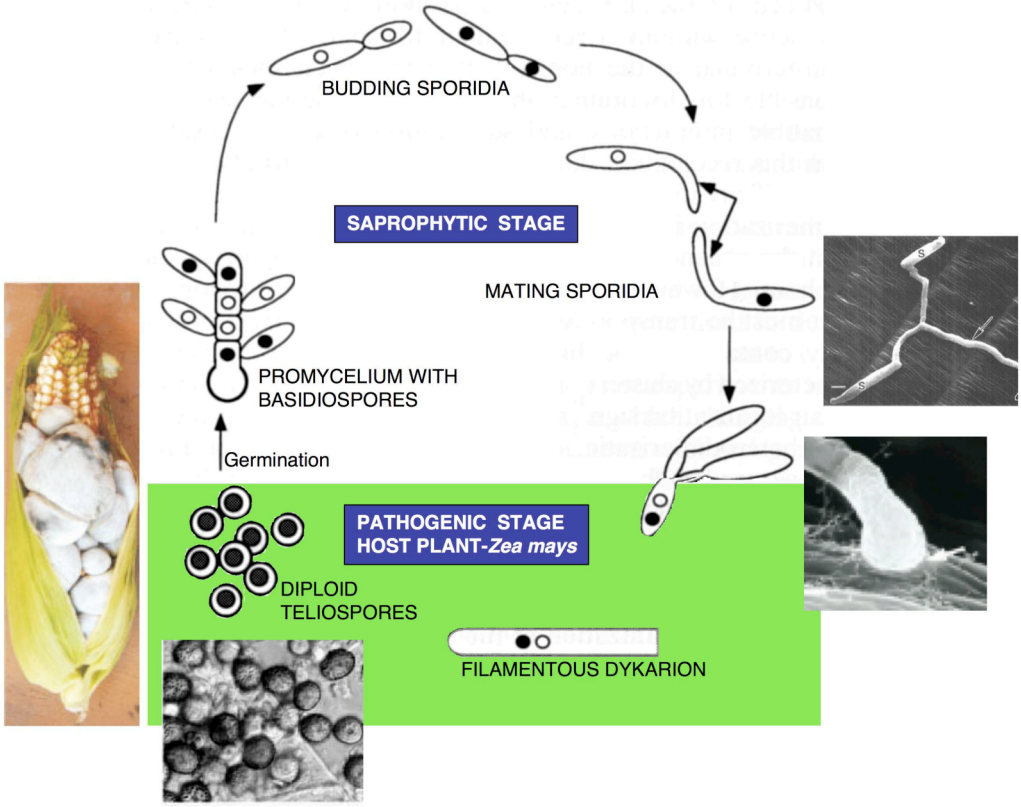


Figure 2: *Ustilago maydis* life cycle. Figure adapted from Casselton and Olesnicky (1998), with pictures from Snetselaar and Mims (1993), Kamper et al.(2006) and Banuett and Herskowitz (1996).

2.2. *Coprinopsis cinerea* life cycle

Coprinopsis cinerea is a model organism commonly used to study developmental processes in the homobasidiomycetous fungi as its life cycle can be completed in the laboratory within 2 weeks (Kues, 2000). It is a typical mushroom, of limited edible value, commonly known as ink cap due to the formation of a inky black fluid that comes from the cap, which is autodigested at maturity (Arora, 1986). There are two distinct mycelial stages in its life cycle: the asexual homokaryon or monokaryon which produces uninucleated asexual spores called oidia, and the fertile dikaryon which is formed upon mating of compatible monokaryons and develop fruiting bodies with sexual spores or basidiospores (Casselton and Zolan, 2002) (Fig. 3).

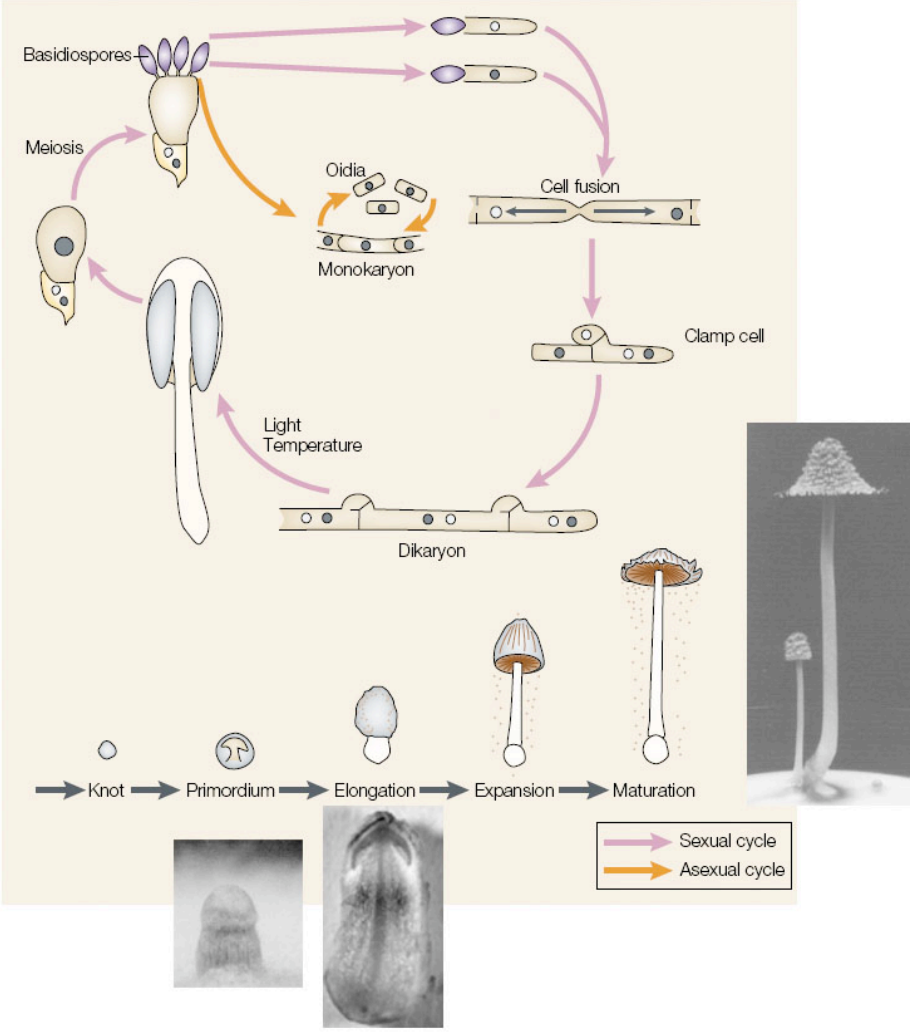


Figure 3: *Coprinopsis cinerea* life cycle. Figure adapted from Casselton and Zolan (2002) with pictures from Kamada (2002) and Wösten and Wessels (2006).

C. cinerea is also a tetrapolar species with two multiallelic mating type loci, *A* and *B*, which must be different to establish a dikaryon. The *A* mating type locus is equivalent to *U. maydis b* genes and is formed by four pairs of divergently transcribed genes that encode the two protein subunits of a heterodimeric regulatory protein. Only one of these four gene pairs is required to be heteroallelic in a cell to trigger *A*-regulated sexual development (Kues et al., 1992). The *B* mating type locus, equivalent to *U. maydis a* locus, consists of three subfamilies of genes, where each subfamily has one gene encoding a pheromone receptor and two genes encoding different pheromones. As it has been described in the *A* locus, only one of these subfamilies is needed to be heteroallelic to control *B*-regulated processes (O'Shea et al., 1998). Once two hyphae from two separate fungi meet, they fuse and compatible *B* genes are required for a nuclear exchange followed by a migration of both nuclei through the established hyphae

of the other homokaryon (Buller, 1931). This nuclear migration is facilitated by the dolipores degradation and can only occur during mating, once cells have nuclei with different *B* factors, septal breakdown is no longer possible (Casselton et al., 1971). Hyphae start to grow as a dikaryon where the *A* locus controls the nuclear pairing, the clamp cell formation, the synchronized nuclear division and the clamp cell septation and the *B* locus is responsible of the clamp cell fusion (Casselton and Olesnicky, 1998) (Fig. 4). Nuclei alternate their position every cell division so that both nuclei have to go through the production of spindles with a different length every cell cycle (Iwasa et al., 1998).

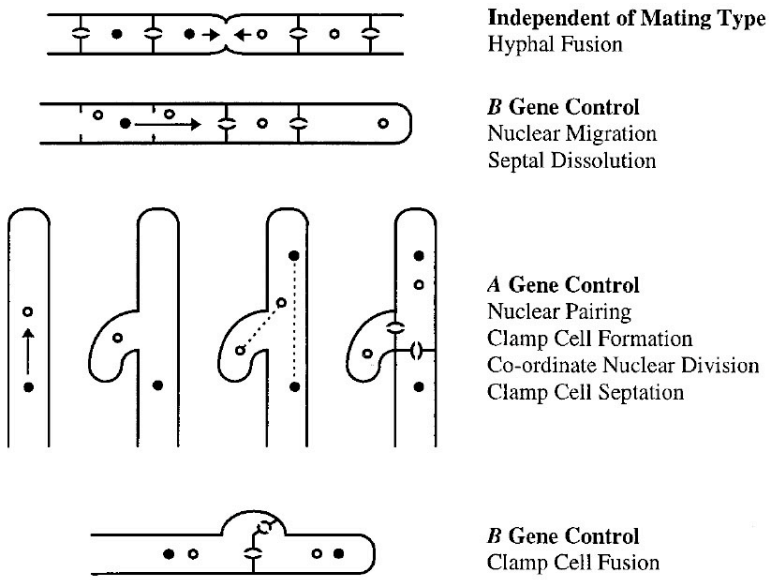


Figure 4: Roles of *A* and *B* genes in dikaryon formation and maintenance in *C. cinerea* (Casselton and Olesnicky, 1998).

After dikaryotization, asexual sporulation is switched off by compatible *A* factors and the initial stages of fruit body differentiation are promoted under certain environmental conditions (Tymon et al., 1992). Fruiting body formation takes seven days under a 12 hours light/12 hours dark regime. In this time, hyphae suffer several changes starting with the formation of hyphal aggregates or knots, followed by tissue differentiation when they become primordia. A few hours before completing the mushroom maturation, primordia go through a strong tissue elongation process. Karyogamy and meiosis takes place in basidia located in the cap. Black basidiospores are released within a brown fluid from the mushroom autolysis a few hours after the cap opening (Navarro-Gonzalez, 2008). The germination of these spores will give raise to a monokaryotic mycelium resuming the life cycle.

3. Cell cycle regulation in yeasts

Cell cycle is composed of four phases: G_1, which is the gap before replication occurs, S, called after DNA synthesis phase, G_2, that stands for gap before mitosis and M where mitosis takes place (Norbury and Nurse, 1992). The transitions from one phase to another occur in an orderly way and are regulated by several proteins. Cell cycle is tightly coupled with cell growth and therefore with cell size and in yeasts two different size-control points exist. In the budding yeast *Saccharomyces cerevisiae* cells must reach a critical size before replicating their genome, being their main control point the G_1-S transition (Neufeld and Edgar, 1998). In *Schizosaccharomyces pombe*, the fission yeast, growth takes place once DNA has been replicated, so its primary cell-size control point is the G_2-M transition (Mitchison and Nurse, 1985). Key regulatory proteins controlling these phase passages are the cyclin-dependent kinases (CDKs), which are serine/threonine kinases that need to bind a cyclin to get active (Pines and Hunter, 1991). In yeasts there is only one Cdk, called Cdc28 in *S. cerevisiae* and Cdc2 in *S. pombe*, which through their association with different cyclins regulate the cell cycle (Nurse and Bissett, 1981; Reed and Wittenberg, 1990). Prior to binding a cyclin, Cdk has to be activated by a Cdk-Activation Kinase (CAK) on Thr169 or Thr167 residues (*S. cerevisiae* and *S. pombe* respectively) (Kaldis, 1999). Cyclins can be gather into two major groups, G1 and B-type or mitotic, depending on which cell cycle transition they control (Pines, 1993). In G_1-S transitions, activated Cdk/cyclin complexes can be inhibited directly by binding to Cdk inhibitors like ScSic1 or SpRum1 or indirectly by targeting their degradation through the ScCdh1-APC or SpSte9-APC complexes. During G_2-M transitions the activated Cdk/cyclin complexes can be inhibited by a phosphorylation on Tyr19 or Tyr-15 (*S. cerevisiae* and *S. pombe* respectively) residues performed by ScSwe1 or SpWee1 protein tyrosine kinases (Rupes, 2002; Russell and Nurse, 1987). Reactivation of Cdk/Cyclin complexes and therefore induction of entry into mitosis is triggered by the ScMih1 or SpCdc25 protein tyrosine phosphatase that dephosphorylates the phosphorylated Tyr residues (King et al., 1994; Russell and Nurse, 1986).

3.1. Cell cycle regulation in *U. maydis*

U. maydis shares similarities with both yeast models. As *S. cerevisiae* is a budding yeast although DNA replication occurs before bud formation, which resembles *S. pombe* (Snetselaar and McCann, 1997) (Fig. 5). Contrary to both yeasts and similar to higher eukaryotes, *U. maydis* cell size is controlled during G_1-S and G_2-M transitions (Perez-Martin et al., 2006).

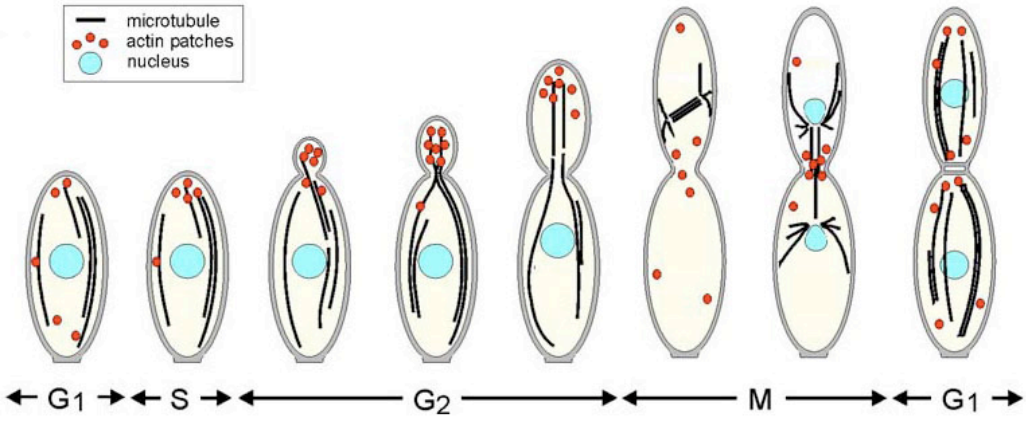


Figure 5: Cell cycle in *U. myadis* coupled to cell morphology. Adapted from Perez-Martin et al., (2006).

U. maydis has one CDK called Cdk1 that can bind to three different cyclins, Cln1 that is a G1 cyclin and Clb1 and Clb2, B-type cyclins (Castillo-Lluva and Perez-Martin, 2005; Garcia-Muse et al., 2003; Garcia-Muse et al., 2004). G_1-S transition is specifically controlled by Clb1-Cdk1 complex. The accumulation of this complex depends on the Cru1-APC complex, which responds to nutritional conditions (Castillo-Lluva et al., 2004). Cln1-Cdk1 complex has also a role in controlling G_1 length as well as the cellular size and the cell morphology (Castillo-Lluva and Perez-Martin, 2005). Once DNA has been replicated, the formation of the bud marks the beginning of the G_2 phase, where the bud will grow until a proper size is reached. The onset of mitosis requires Clb1-Cdk1 and Clb2-Cdk1 complexes, but only the last one determines the length of G_2: low levels of Clb2 induce a G_2 delay that results in cells with elongated buds while overexpression of *clb2* results in short cells that divide by septation (Garcia-Muse et al., 2004). Clb2-Cdk1 tight regulation is controlled via the inhibitory phosphorylation of Cdk1 by Wee1, as the phenotype observed after *wee1* down-regulation resembles the one observed after *clb2* overexpression and vice versa (Sgarlata and Perez-Martin, 2005b). Removal of the inhibitory phosphorylation at the onset of mitosis is done by the Cdc25 phosphatase and therefore *cdc25* overexpression

results in a *wee1*-ablated phenocopy and the other way around (Sgarlata and Perez-Martin, 2005a). Like in other yeasts, *U. maydis* has a 14-3-3 like protein named Bmh1, that binds to Cdc25 and inhibits its activity (Mielnichuk and Perez-Martin, 2008). Other regulators characterized in *U. maydis* that have a role in this transition are Plk1, a polokinase-like protein and Hsl1 that belongs to the Nim1/Cdr1 protein family. Plk1 has shown to have a role in Cdc25 activation, although a control over Wee1 activity could not be dismissed. For Hsl1 a role in controlling the length of G_2 has been proposed (Mielnichuk, 2007) (Fig. 6).

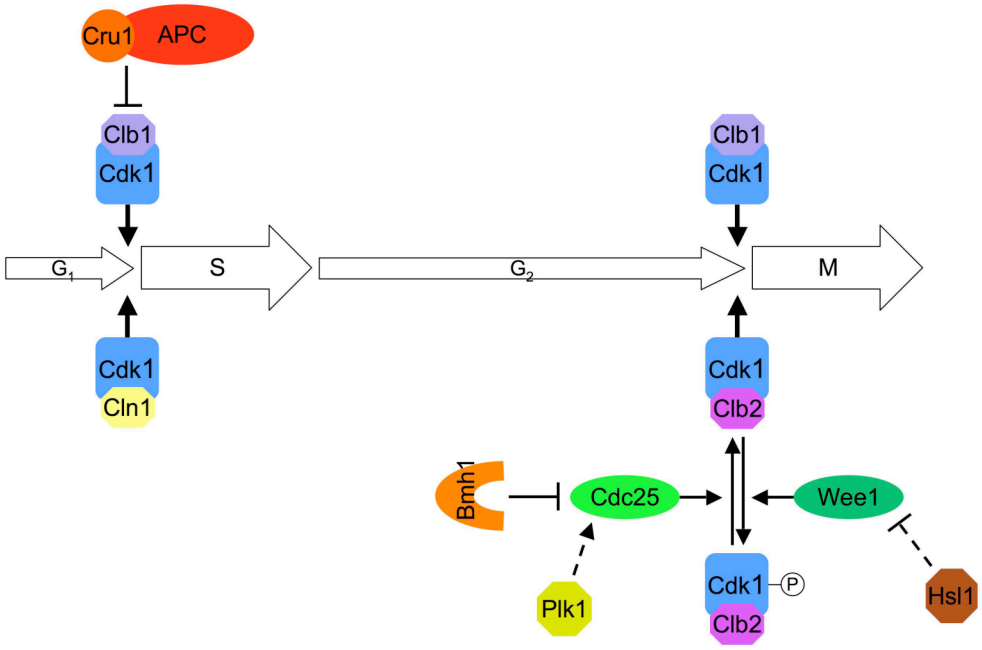


Figure 6: Cell cycle regulation *in U. maydis*

3.2. Cell cycle and virulence in *U. maydis*

Virulence and sexual development are closely intertwined in *U. maydis*. A prerequisite for both processes is the mating of two compatible budding haploid cells to give rise to a dikaryotic filament. This process implies morphological and genetic changes and therefore an accurate control of the cell cycle and the morphogenesis is essential. Poor nutrient conditions, as the ones expected to occur on the leaf surface, lead to a stimulation of *prf1* expression (Castillo-Lluva and Perez-Martin, 2005). Prf1 controls the transcription of the *a* and *b* genes (Hartmann et al., 1996). The *a* genes code for a pheromone-receptor system and trigger the formation of conjugative tubes and the cell fusion (Spellig et al., 1994). As it happens in other fungal systems pheromone recognition blocks cell cycle progression in order to prepare the mating partners for

conjugation, which in *U. maydis* is translated into a G_2 cell cycle arrest (Garcia-Muse et al., 2003). Besides, sensing of the pheromone activates a MAPK cascade that leads to the activation of several genes including the *b* genes (Kaffarnik et al., 2003). Fusion of the conjugation tubes brings about the formation of a dikaryotic cell with a strong polar growth in which the cell cycle will remain arrested until it enters inside the plant (Snetselaar and Mims, 1994). Also, upon cellular fusion, the interaction between the two subunits that compose the b-factor, bW and bE, occurs and therefore all the b-controlled processes are activated. Within these processes are the maintenance of the filamentous growth and the cell cycle arrest.

G_2 cell cycle arrest induced by the b-heterodimer complex is dependent on the inhibitory phosphorylation of Cdk1 Tyr15 residue, as upon b-induction, Tyr^{15}P-Cdk1 levels increase greatly and overexpression of a Cdk1 refractory to inhibitory phosphorylation under b-expressing conditions leads to the formation of multinucleated filaments (Mielnichuk et al., 2009). Wee1 and Cdc25 trigger the accumulation of Tyr^{15}P-Cdk1. Wee1 involvement in this cell cycle arrest coupled to the induction of a strong polar growth correlates with the model proposed by Kellog (2003), where Wee1-related kinases are suggested to monitor the total polar growth that occurs in the cell. In Cdk1 regulation upon b-proteins induction, Wee1 is overexpressed to trigger Cdk1 phosphorylation but at the same time Cdc25 is sequestered in the cytoplasm to ensure the cell cycle arrest. Bmh1 is the protein in charge of binding to Cdc25 and triggering its retainment. Previously to this binding, Cdc25 needs to be phosphorylated and Chk1, a DNA damage response kinase is involved in this step (Mielnichuk and Perez-Martin, 2008), which creates a recognition site for Bmh1. Cells defective in Chk1 and expressing compatible b-proteins are impaired in their ability to arrest the cell cycle since filamentous cells present several nuclei (Mielnichuk et al., 2009). G_2 cell cycle arrest does not fully rely on Chk1, since Chk1 is only transiently activated, so still more components that take part in this process have to be characterized (Fig. 7).

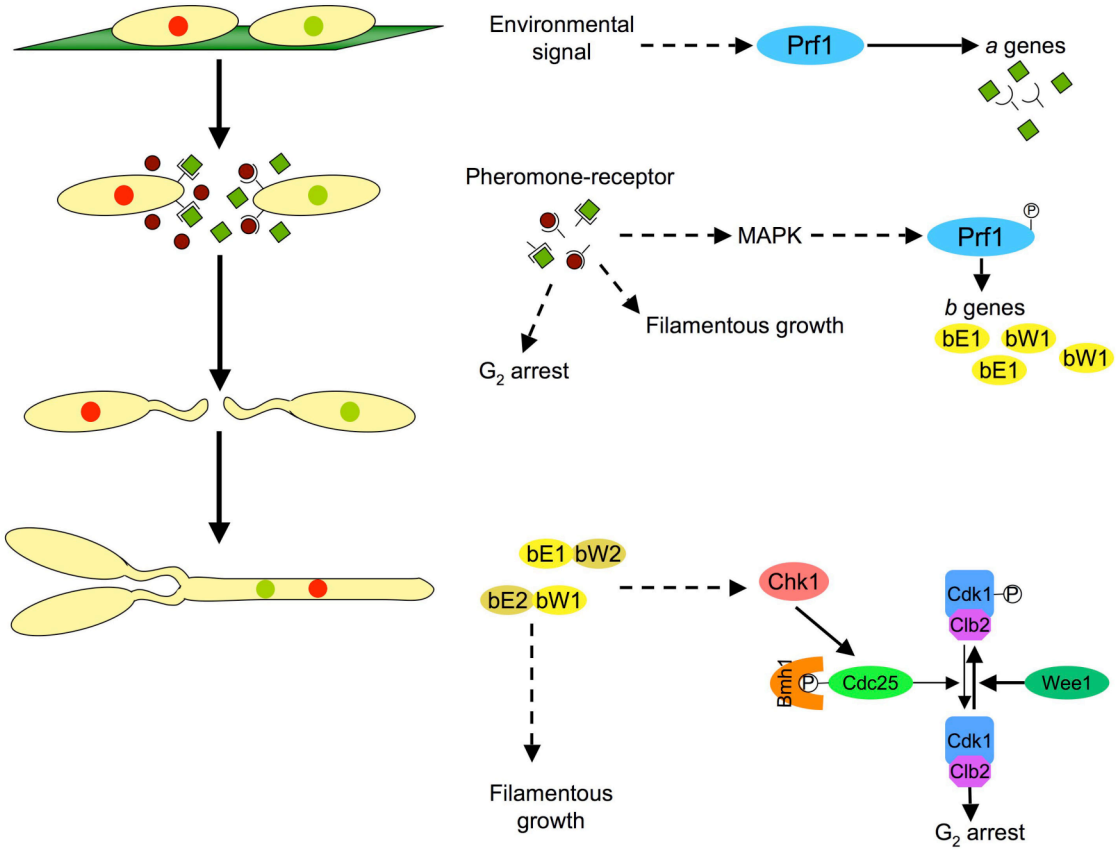


Figure 7: Cell cycle regulation during pathogenesis in *U. maydis*

4. The DNA damage response

Cells are subjected to continuous assaults on their genome caused either by environmental or internal agents; therefore evolution has overlaid the core cell-cycle machinery with a series of surveillance pathways named cell-cycle checkpoints that ensure an accurate genome transfer to the next generation. One of these checkpoints is the so-called DNA damage response (DDR), whose role is to detect damaged DNA and coordinate its repair with the cell cycle progression (Abraham, 2001; Nyberg et al., 2002). The current idea is that DNA damage checkpoints pathways work under normal conditions and are amplified upon an increase in DNA damage (Sancar et al., 2004). The DNA damage checkpoint pathway involves three major groups of proteins: sensors, signal transducers and effectors.

Sensors are proteins that recognize damaged DNA and signal the presence of the abnormalities. Among these sensors several protein complexes are found: the MRN/MRX complex, formed by Mre11 Rad50 and Nbs1 or Xrs2 depending on the organism, the 9-1-1 clamp that comprises Rad9, Hus1 and Rad1 proteins and the Ku

heterodimer that includes Ku70 and Ku80 proteins (Falck et al., 2005). The MRN protein complex is involved in ATM recruitment to DNA double-strand breaks (Carson et al., 2003). It binds to the DNA through Mre11 protein and dimerizes with another MRN complex through Rad50 (van den Bosch et al., 2003).The 9-1-1 clamp is associated with ATR activation in response to single-stranded DNA (Navadgi-Patil and Burgers, 2009). Is a heterotrimeric ring-shaped molecule that is related in structure and sequence to the Proliferating Cell Nuclear Antigen (PCNA), a replicative sliding clamp (Parrilla-Castellar et al., 2004). The 9-1-1 complex recognizes a DNA strand that is adjacent to a RPA coated ssDNA region (Majka et al., 2006). Ku70/Ku80 heterodimer is responsible for the DNA-PKcs recruitment to DSBs (Gottlieb and Jackson, 1993). It forms a pseudo-symmetrical molecule with a ring inside that encircles double stranded DNA.

The sensors transmit the signal to protein kinases, which relay and amplify the damage signal by phosphorylating other downstream target proteins. The protein kinases that form this group belong to the PhosphatidylInositol 3-Kinase related Kinases (PIKKs), and some members of this family are ATM, ATR and DNA-PKcs. ATM and ATR are well conserved among eukaryotes while DNA-PKcs is only present in vertebrates (Jackson, 2002). PIKKs have a significant sequence homology between them. They phosphorylate Ser or Thr residues followed by Gln and target an overlapping set of substrates that promote cell cycle arrest and DNA repair (Cimprich and Cortez, 2008).

ATM was identified in the ‘90s as the gene defective in AT (ataxia-telangiectasia) syndrome, which causes neurodegenerative disorders and cancer predisposition (Savitsky et al., 1995). Cells lacking ATM presented chromosomal instability and sensitivity to ionizing radiation and radiomimetic drugs. These cells are defective at G_1-S, S and G_2-M cell cycle checkpoints upon radiation-induced damage (Abraham, 2001). Upon DSBs formation ATM is recruited to the sites of damage by the MRN complex, which phosphorylates ATM through its interaction with Nbs1 (Abraham and Tibbetts, 2005).

ATR (ATM and Rad3-related) is related to the Seckel syndrome, a rare human disease characterized by growth retardation and microcephaly (O'Driscoll et al., 2003). Disruptions in ATR pathway cause genomic instability. ATR is activated in response to the presence of stretches of ssDNA. In the cell, replication protein A (RPA) coats most forms of ssDNA (Fanning et al., 2006), therefore the structure that causes the activation

of the ATR checkpoint pathway is ssDNA bound to RPA, with an adjacent region of dsDNA that presents a 5' junction. This structure is generated during replication, in end resection at DSBs, at telomeres and during nucleotide-excision repair (Cimprich and Cortez, 2008). ATR recognition of RPA-ssDNA depends on ATR-interacting protein (ATRIP) whose association is essential for ATR activity (Ball and Cortez, 2005). For ATR activation in some cases the TOPoisomerase-Binding Protein-1 (TopBP1) is needed (Delacroix et al., 2007). In these cases the structures that cause ATR activation are also recognized by 9-1-1 clamp which recruits TopBP1 (Lee et al., 2007). TopBP1 contains an ATR activation domain that when interacting with ATR can phosphorylate it.

DNA-PKcs plays a critical role in Non-Homologous End-Joining (NHEJ) in stabilizing DSBs ends, preventing end resection and promoting the end rejoining. The interaction with the Ku heterodimer is through a domain present in Ku80. This interaction stabilizes the DNA-PKcs binding to the DNA and activates it to initiate NHEJ (Ciccia and Elledge, 2010; Durocher and Jackson, 2001).

Finally, effector kinases usually regulate by phosphorylation downstream targets to prevent a cell cycle progression. Chk1 and Chk2/Rad53 form this group, serine-threonine kinases required for the cell cycle arrest in response to DNA damage. As downstream kinases, they are phosphorylated in an ATR/ATM-dependent manner. Chk1 and Chk2 phosphorylation is triggered through mediators, proteins that simultaneously associate with transducers and effectors. Within these mediator proteins, MDC1, 53BP1, Claspin and BRCA1 with their respective yeast homologues are found (Nyberg et al., 2002; Sancar et al., 2004). Activation of Chk1 and Chk2 triggers the phosphorylation of Cdc25, which results in the creation of a 14-3-3 binding site in the case of Chk1 or a mark for its degradation in the case of Chk2/Rad53 (Abraham, 2001). As a result of this phosphorylation, Cdc25 is either sequestered in the cytoplasm or degraded or both. The inactivation of Cdc25 as previously seen leads to a cell cycle arrest (Sanchez et al., 1997) (Fig. 8).

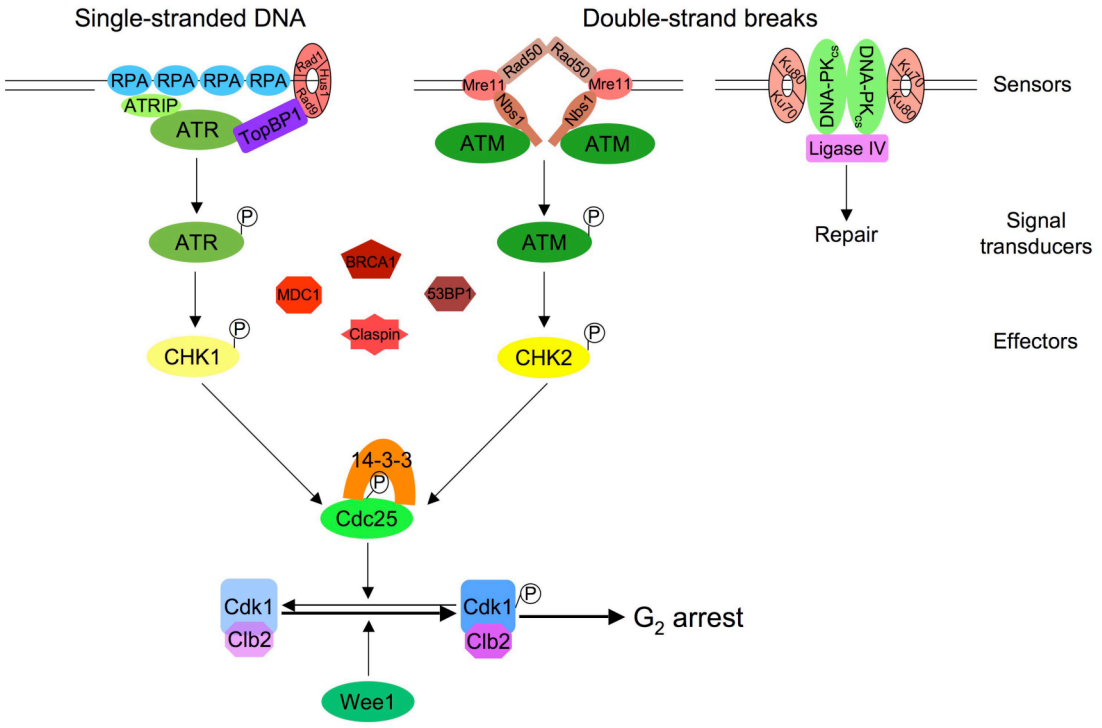


Figure 8: DNA damage response pathways

4.1. DNA damage checkpoint pathway in *U. maydis*

U. maydis has been used as a model system for studying DNA repair following UV or ionizing radiation for many years, indeed, the first DNA repair mutants isolated among the eukaryotes were obtained in *U. maydis* (Holliday, 1965). DNA repair pathways, specially the homologous recombination one, has been studied and characterized in the past years (Holloman et al., 2008). Interestingly very little is known about the DNA damage signaling cascades in *U. maydis*. Among the sensors and the signal transducers, homologues for the MRN complex, Ku heterodimer, 9-1-1 clamp, RPA, TopBP1, ATM and ATR have been identified by sequence homology and some of them like RPA and Rec1 (Rad1 homologue) have been characterized (Holliday et al., 1976; Sanchez-Alonso and Guzman, 2008; Thelen et al., 1994; Yang et al., 2005). No homologues for the DNA-PKcs, ATRIP or the mediator proteins have been identified through homology search. And within the effectors only Chk1 has been characterized in response to DNA damage since no Chk2/Rad53 homologue is present in *U. maydis*. Chk1 gets phosphorylated in response to several DNA damage insults and its deletion impairs the cells to adjust cell cycle to the presence of DNA damage (Perez-Martin, 2009). The 14-3-3-like protein, Bmh1 is involved in DNA damage response suggesting

a conservation of the described DNA damage checkpoint pathway in *U. maydis* (Mielnichuk and Perez-Martin, 2008) (Fig. 9).

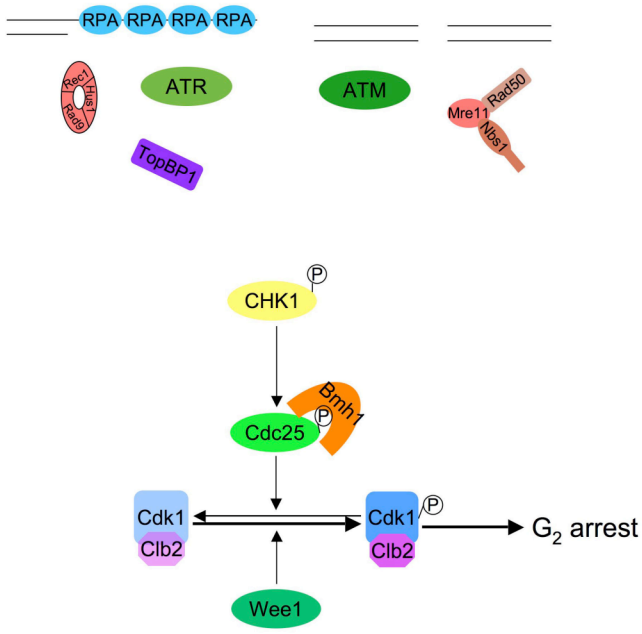


Figure 9: DNA damage response pathways in *U. maydis*

5. Telomeres

Genome organization into linear chromosomes requires mechanisms to protect and ensure that their termini are fully duplicated when the chromosome itself is replicated. For this, eukaryotic cells have developed telomeres and telomerase. Telomeres are specialized nucleoprotein complexes that allow cells to distinguish chromosome ends from sites of DNA damage. In mammals, this protein complex is called shelterin and in yeasts is known as telosome. And telomerase is the enzyme that lengthens terminal regions of telomeric DNA (Denchi, 2009).

In eukaryotes telomeric DNA consists of tandem repeats of G-rich short sequences like TTAGGG (Greider, 1996). Rather than being blunt-ended, telomeres terminate with a 3' single-stranded overhang that invades the duplex telomeric DNA and anneals to the complementary C-strand, forming a secondary structure termed t-loop (Griffith et al., 1999). Next to the terminal telomeric repeats, subtelomeric sequences can be found. These sequences are also tandemly repeated and shared between telomeres, but differ greatly between species (Blackburn, 2001)

Telomerase, which specifically elongates telomeres, is a ribonucleoprotein enzyme that by using a RNA-templated cast adds tandemly repeated telomeric

sequences to maintain the length of chromosomal ends. Is formed by a highly conserved reverse transcriptase and an associated template RNA (Greider and Blackburn, 1989; Nakamura et al., 1997). Usually telomerase contains additional proteins that are not required for catalysis *per se*, but whose presence is required for correct telomere maintenance. These proteins are two accessory proteins named Est1 and Est3 in *S. cerevisiae* and EstA and EstB in humans that bind the RNA component, and a RNA cap known as dyskerin or Sm proteins (Humans and *S. cerevisiae* respectively) (Smogorzewska and de Lange, 2004).

The composition of the shelterin complex in humans and the telosome in yeasts varies but some essential features are conserved. The proteins that form both complexes bind to the telomeric repeats region. In both complexes TRF-like proteins that bind double stranded DNA are found, TRF1 and TRF2 in humans and Taz1 in *S. pombe*, which are essential for the telomere length control and prevention of end-joining reactions (Bae and Baumann, 2007; Ferreira and Cooper, 2001). An important characteristic of these proteins is that they are necessary for the t-loop formation at telomeres (Tomaska et al., 2004). Also a single stranded DNA binding protein, which is located at the 3' single-stranded G overhangs is present both in humans and *S. pombe*. This protein named Pot1 (Protection Of Telomeres) protects the telomeres from degradation and in humans regulates telomerase activity (Baumann and Cech, 2001). Apart from these proteins, several interacting proteins complete the shelterin or telosome complex like: TIN2, TPP1 and Rap1 in humans or Poz1, Tpz1 and Rap1 in fission yeast (de Lange, 2005; Kanoh and Ishikawa, 2001) (Fig. 10).

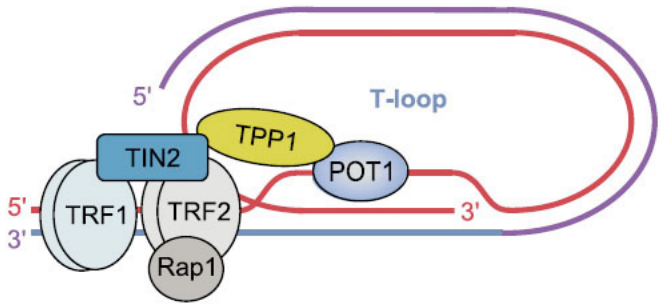


Figure 10: Scheme picturing the t-loop formation at telomeres and the structure of the shelterin complex in humans (De Cian et al., 2008).

In addition to the shelterin components, non-exclusive telomeric proteins transiently associate with the telomeres. Most of these proteins are involved in DNA damage signaling and repair, some of them are: MRN complex, DNA-PKcs and Ku heterodimer, Rad3 and Rad26 (ATR and ATRIP homologues in fission yeast), ATM and BLM and WRN, RecQ helicases, and Rad51, which are involved in the homologous recombination pathway (Palm and de Lange, 2008).

5.1. Telomeres and the DNA damage response

An essential role of the proteins associated to telomeres is to avoid that the DNA damage signaling pathways recognize telomeres as damaged DNA. ATM or ATR can transmit these signals and therefore are targets of suppression. In humans deletion of TRF2 triggers the activation of the DNA damage response through the MRN complex-ATM pathway (Deng et al., 2009). ATM inhibition at telomeres has been proposed to be controlled by TRF2, which can bind to ATM and inhibit its phophorylation and therefore the activation of the DNA damage response pathway (Karlseder et al., 2004). The mechanism of ATR activation relies on the recruitment of RPA to single-stranded G-overhangs. At the telomere RPA competes against POT1 for the single-stranded DNA, competition that under normal conditions POT1 wins owing to the fact that it belongs to the shelterin complex (Denchi and de Lange, 2007) (Fig. 11).

In *S. pombe*, 9-1-1 clamp, Rad3 and Rad26 (ATR and ATRIP homologue proteins respectively) are bound to telomeres and are important for telomere maintenance (Nakamura et al., 2002). The inhibition of a DNA damage response that eventually would lead to Chk1 phosphorylation followed by a cell cycle arrest is triggered by Pot1, Ccq1, which is a Pot1 interacting protein required for telomerase recruitment, and Taz1. These proteins create a chromatin-privileged region on the chromosome that blocks the transduction of an active checkpoint signal by preventing the stable association of 53BP1 homologue, Crb2 with telomeres (Carneiro et al., 2010). How Tel1, ATM homologue in fission yeast, is inhibited at telomeres is still unknown.

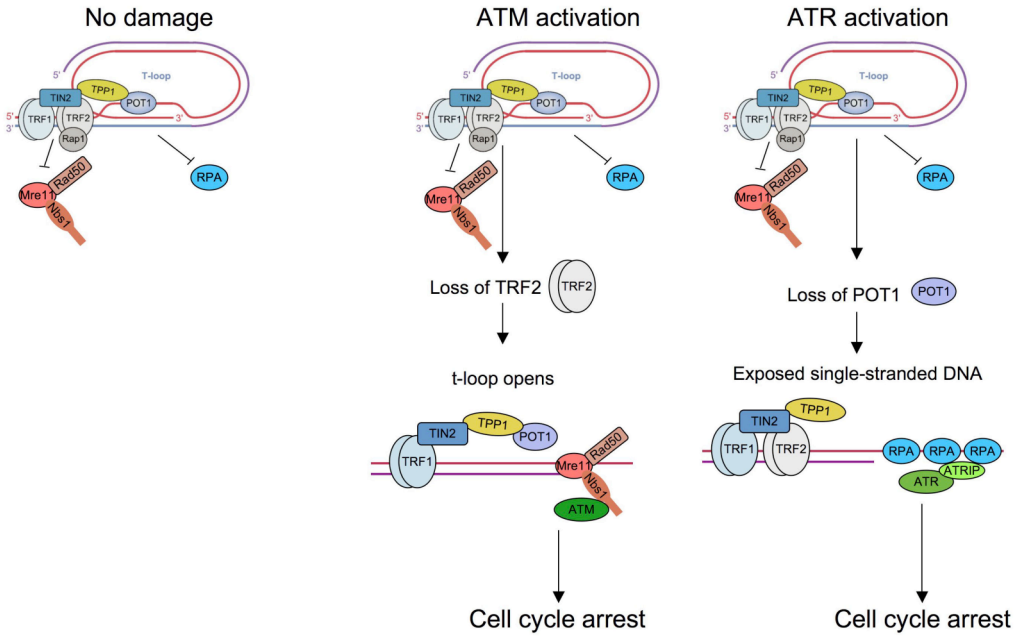


Figure 11: Mechanisms that lead to the activation of the DNA damage response pathway. Adapted from de Cian et al., (2008).

5.2. Telomeres and DNA repair

The shelterin complex, not only suppresses the activation of the DNA damage response, but also is essential to repress inappropriate repair reactions at chromosome ends. Base excision repair, and mismatch repair are presumably used to maintain the TTAGGG repeat sequence, while NHEJ or HR could have disastrous outcomes (de Lange, 2005). Activation of NHEJ would result in end-to-end chromosome fusions forming dicentric chromosomes, which eventually could not be well segregated in mitosis. The shelterin component TRF2 is involved in suppression of the NHEJ pathway. When TRF2 is absent, t-loop formation is not formed correctly and 3' overhangs are removed by an exonuclease. This leads to DSBs formation, and binding of the Ku heterodimer together with the DNA-PKcs that trigger the repair of the broken ends through the activity of the Ligase IV (Denchi, 2009). An interesting player in this pathway whose activity still has to be elucidated is Ku heterodimer. Ku proteins seem to have contradictory roles in telomeres, they are involved in NHEJ and at the same time they have been described as telomere protectors against end-to-end fusions (Fisher and Zakian, 2005). The current interpretation of this paradox is that the shelterin might restraint the activities of the Ku heterodimer (de Lange, 2009). Activation of the other type of DNA repair mechanism, homologous recombination at telomeres would generate aberrant telomere length, telomere deletions, and translocations. TRF2 is also involved in suppressing the homologous recombination pathway at telomeres. Even

more, TRF2 and Ku70 seem to act in a redundant manner to prevent recombination at telomeres (Baumann and Cech, 2000) (Fig. 12).

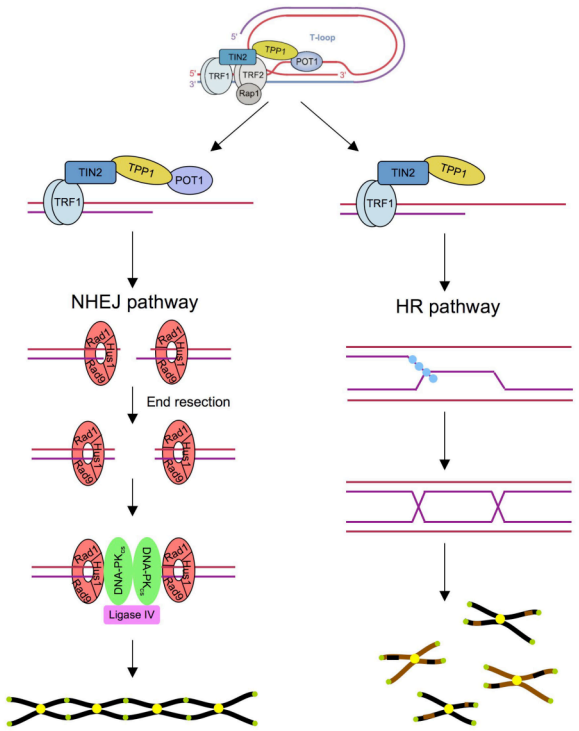


Figure 12: Activation of the DNA repair pathways in telomeres. Adapted from de Cian et al., (2008).

5.3. Telomeres in *U. maydis*

The information about telomere organization in *U. maydis* is very scant. Telomeric repeats have been identified and their sequence, TTAGGG, is the same that is present among most eukaryotes. Adjacent to the telomeric repeats two repetitive sequences found at several chromosomal ends and polymorphic in length are present. These sequences are known as *Ustilago maydis* Telomere-Associated Sequences or *UTAS*. *UTASa* is highly conserved and is mostly located at chromosomal ends, while *UTASb* is less conserved and can be found throughout the genome (Sanchez-Alonso and Guzman, 1998). These are all the experimental data available about *U. maydis* telomeres. The rest is only a prediction of the telomerase and the shelterin components identified by sequence homology through the *U. maydis* genome annotated database. Within these data some of the telomerase components have been identified: the reverse transcriptase catalytic subunit, the dyskerin and the Est1. Contrary to telomerase components identification, only the DNA binding components of the shelterin have been found: Pot1 and Trf1 (Sanchez-Alonso and Guzman, 2008).

Introducción en Español

1. Propósito del trabajo

El dimorfismo es la capacidad de algunos hongos para cambiar su forma de crecimiento de levadura unicelular a filamento multicelular. Este fenómeno es explotado ampliamente por hongos patógenos de animales y plantas, donde a raíz de un contacto con el hospedador se induce un cambio en la forma de crecimiento (Rooney and Klein, 2002). Los cambios en el medio ambiente generan una variedad de señales que son percibidas por el hongo dimórfico induciendo el cambio morfológico como una forma de adaptación a las nuevas condiciones. De manera que la identificación de las señales y de los procesos que finalmente llevan al cambio morfológico es necesaria para encontrar nuevas dianas que impidan la infección. *Ustilago maydis* cambia de un estado de levadura saprófita a un filamento patógeno en respuesta a las señales de la planta y a su contenido nuclear. El crecimiento filamentoso solo ocurre en el interior de la planta de maíz, lo que indica que una señal proveniente de la planta viva es esencial para desencadenar este comportamiento (Nadal et al., 2008). Dimorfismo, desarrollo sexual y virulencia son procesos íntimamente asociados en los ciclos de vida de los hongos dimórficos, y en *U. maydis* todos estos cambios están asociados a la regulación del ciclo celular. La activación del programa de virulencia lleva a una parada del ciclo celular, la cual recientemente se ha visto que está provocada por una quinasa de checkpoint de daño en el ADN (Mielnichuk et al., 2009). La idea de que una conocida quinasa de la ruta de respuesta a daño en el ADN estuviera involucrada en la parada de ciclo celular provocada por una señal de desarrollo, planteó varias preguntas: ¿La parada de ciclo celular es una consecuencia de la presencia de daño en el ADN durante los cambios morfológicos? ¿Cómo se activa la quinasa de checkpoint?

Durante mucho tiempo *U. maydis* se ha estado utilizando como un organismo modelo en el estudio de los procesos de reparación del ADN. Las estructuras de Holliday, que son esenciales en los procesos de recombinación homóloga (HR) y cuya presencia está conservada de procariotas a mamíferos, fueron descritas en *U. maydis* por Robin Holliday en los años 60. Desde entonces, avances significativos han motivado un cambio en la consideración de *U. maydis* hacia un organismo simplificado en el que estudiar las rutas de respuesta a daño en el ADN y de reparación del ADN con gran similitud a humanos. Un buen ejemplo de esto es el gen *brh2*. *brh2* es el homólogo del BRCA2 humano y, al igual que en mamíferos, es esencial para el mantenimiento de la estabilidad genómica y para la eficiencia de los procesos de reparación y recombinación

(Kojic et al., 2002). Otros ejemplos incluyen la organización de los microtúbulos del citoesqueleto durante la mitosis o el crecimiento polar y la eliminación de la membrana nuclear durante mitosis, ambos son procesos más cercanos a mamíferos que a levaduras (Steinberg and Perez-Martin, 2008). Al contrario que la ruta de reparación por recombinación homóloga ampliamente caracterizada, la ruta de respuesta a daño en el ADN en *U. maydis* se desconoce. Debido a esto, *U. maydis* se utilizó para responder a las preguntas anteriormente mencionadas y para caracterizar esta ruta de respuesta a daño en el ADN.

2. Características especiales de los basidiomicetos

Los basidiomicetos son una división dentro del reino de los hongos que incluye a setas, poliporos, patógenos de plantas como *Ustilago maydis* y patógenos humanos como *Cryptococcus* (James et al., 2006). Los basidiomicetos se pueden distinguir del resto de los hongos porque la meiosis tiene lugar en células especiales llamadas basidios y está seguida inmediatamente por la producción de cuatro basidiosporas. Otra característica compartida por la mayoría de los basidiomicetos es la presencia de dos fases en su ciclo de vida: una fase haploide homocariótica que normalmente es asexual pero que puede producir esporas asexuales y una fase heterocariótica en la cual cada célula contiene dos núcleos de distinto tipo sexual. Está segunda fase es conocida como dicarionte y proviene de la anastomosis entre dos hifas o células haploides de distinto origen, normalmente es el prerrequisito para la reproducción sexual y es el estado micelial predominante en la naturaleza. Los dos núcleos permanecen individuales durante las divisiones somáticas de las células gracias a la formación de fíbulas, y únicamente después de formar el cuerpo fructífero se fusionan en células reproductivas especializadas, los basidios, donde inmediatamente sufren meiosis (Casselton, 1978; Fincham et al., 1979). Las fíbulas garantizan una distribución nuclear correcta que mantiene la presencia de dos núcleos diferentes por célula. Previamente a la división celular, la fíbula se desarrolla en un lado de la célula apical y uno de los núcleos migra a su interior mientras el otro permanece en la célula principal. Los dos núcleos se dividen sincrónicamente y los septos se forman perpendicularmente al plano del huso mitótico. Seguidamente la fíbula se fusiona con la célula subapical y su núcleo migra a esta célula (Casselton, 1978) (Fig. 1).

2.1. Ciclo de vida de *Ustilago maydis*

Ustilago maydis es un hemibasidiomiceto también conocido como el carbón del maíz (Christensen, 1963). Su rango de hospedadores es muy pequeño infectando únicamente al maíz (*Zea mays*) y al teosinte (*Zea mays* subsp. *parviglumis*) (Bolker, 2001). Es un hongo dimórfico cuyo ciclo de vida está dividido en dos fases: una fase asexual saprófita durante la cual crece como una levadura de gemación y una fase sexual patógena donde crece como un dicarionte (Perkins, 1949). El cambio de la fase saprófita a la patógena ocurre después del apareamiento de dos células compatibles, y en la naturaleza esté proceso tiene lugar en la superficie de la planta (Snetselaar and Mims, 1992). *U. maydis* es una especie tetrapolar heterotálica, lo que significa que la compatibilidad recae en dos loci que determinan el tipo sexual llamados *a* y *b* que al menos tienen dos alelos por cada locus (Raper, 1953). El locus *a* tiene dos alelos, *a1* y *a2* y controla la fusión celular y el crecimiento filamentoso a través de un sistema basado en el reconocimiento de una feromona por un receptor (Banuett and Herskowitz, 1989; Bolker et al., 1992; Holliday, 1974). El locus *b* es multialélico y codifica dos factores de transcripción homeodominio, *bE* y *bW*, que controlan la patogenicidad del dicarionte resultante de la fusión celular (Banuett, 1995; Puhalla, 1970). El filamento infectivo que surge de la fusión de dos tubos conjugativos con genes *a* y *b* compatibles forma una estructura de tipo apresorio para penetrar en la planta (Snetselaar and Mims, 1992). Este apresorio, morfológicamente similar al resto de los apresorios formados por otros hongos patógenos, no está melanizado, sugiriendo que probablemente otro mecanismo como la producción de enzimas líticas podría cooperar con la fuerza mecánica de este, aunque esto todavía no está claro (Garcia-Pedrajas et al., 2004). Una vez que el hongo entra dentro de la planta crece inter e intracelularmente a través de la epidermis y el parénquima hasta llegar a los haces vasculares (Snetselaar and Mims, 1994). Prolifera como un dicarionte formando una amplia red de hifas e induciendo la formación de tumores. Los síntomas en la planta comienzan con la aparición de clorosis y manchas de antocianina las cuales están asociadas con la respuesta de la planta al estrés producido por la penetración del hongo, y están seguidas por la hiperplasia e hipertrofia de los tejidos que terminarán en la formación de tumores (Callow and Ling, 1973). Dentro de los tumores, las hifas se incrustan en una matriz mucilaginosa, y los núcleos se fusionan a la vez que la hifa se fragmenta. Estos fragmentos sufren varios cambios morfológicos hasta formar las teliosporas (Snetselaar and Mims, 1994). Al

romperse el tumor, las teliosporas son dispersadas por el viento y su posterior germinación produce cuatro basidiosporas haploides, cerrando de está manera el ciclo de vida (Ingold, 1983) (Fig. 2).

2.2. Ciclo de vida de *Coprinopsis cinerea*

Coprinopsis cinerea es un organismo modelo comúnmente usado para el estudio de los procesos de desarrollo de los homobasidiomicetos ya que su ciclo de vida se puede completar en el laboratorio en dos semanas (Kues, 2000). Es una seta típica, de limitado valor comercial, comúnmente conocida como seta de tinta debido a la formación de un liquido negro, que recuerda a la tinta, en el sombrero, como resultado de la autodigestión de este una vez que madura (Arora, 1986). Durante su ciclo de vida pasa por dos estados miceliales distintos: el homocarionte asexual o monocarionte que produce esporas uninucleadas asexuales llamadas oidios y el dicarionte fértil, el cual se forma a partir del apareamiento de dos monocariontes compatibles y desarrolla cuerpos fructíferos con esporas sexuales o basidiosporas (Casselton and Zolan, 2002) (Fig. 3).

C. cinerea también es una especie tetrapolar, con dos loci multialélicos que determinan el tipo sexual, *A* y *B*, los cuales deben ser distintos para establecer un dicarionte. El locus *A* es equivalente a los genes *b* de *U. maydis* y está formado por cuatro pares de genes transcritos divergentemente que codifican las dos subunidades proteínicas de una proteína reguladora heterodimérica. Únicamente uno de estos cuatro pares de genes tiene que ser heteroalélico en una célula para activar el desarrollo sexual regulado por los genes *A* (Kues et al., 1992). El locus *B*, equivalente al locus *a* de *U. maydis*, consiste en tres subfamilias de genes, donde cada subfamilia tiene un gen que codifica un receptor para feromonas y dos genes que codifican feromonas distintas. Como se ha mencionado previamente para el locus *A*, únicamente una de estás tres subfamilias tiene que ser heteroalélica para activar los procesos regulados por el locus *B* (O'Shea et al., 1998). Una vez que dos hifas provenientes de hongos diferentes se encuentran, se fusionan y los genes *B* compatibles son necesarios para que el intercambio nuclear seguido por la migración de los núcleos a través del homocarionte opuesto ocurra (Buller, 1931). Está migración nuclear está facilitada por la degradación de los doliporos y solamente puede ocurrir durante el apareamiento, una vez que las células tienen dos núcleos con distintos genes *B*, la rotura de los septos no es posible (Casselton et al., 1971). Las hifas empiezan a crecer como un dicarionte donde el locus *A* controla el emparejamiento de los núcleos, la formación de las fíbulas, la división

nuclear sincrónica y la formación de los septos, mientras que el locus *B* es el responsable de la fusión de las fíbulas (Casselton and Olesnicky, 1998) (Fig. 4). Los núcleos alternan su posición en cada división celular, de manera que ambos núcleos tienen que producir husos mitóticos de distinta longitud en cada ciclo celular (Iwasa et al., 1998).

Después de la dicariotización, la presencia de factores *A* compatibles reprime la esporulación asexual e induce los primeros pasos en la diferenciación que tiene lugar en la formación de cuerpos fructíferos bajo determinadas condiciones ambientales (Tymon et al., 1992). La formación de los cuerpos fructíferos maduros tarda 7 días bajo un régimen de 12 horas de luz/12 horas de oscuridad. Durante este tiempo las hifas sufren varios cambios comenzando con la formación de agregados de hifas o nudos seguidos por la diferenciación de los tejidos en los primordios. Unas pocas horas antes de completar la maduración de la seta, los primordios sufren una fuerte elongación de los tejidos. La cariogamia y la meiosis ocurre en los basidios del sombrero. Las basidiosporas negras son liberadas dentro de un liquido parduzco proveniente de la autolisis de la seta tan solo unas horas después de la apertura del sombrero (Navarro-Gonzalez, 2008). La germinación de estas esporas dará lugar a un micelio monocarionte completando así su ciclo de vida.

3. Regulación del ciclo celular en levaduras

El ciclo celular está formado por cuatro fases: G_1 o intervalo 1 que ocurre antes de la replicación, S, donde tiene lugar la síntesis de ADN, G_2 o intervalo 2, previo a la mitosis y M, donde ocurre la mitosis (Norbury and Nurse, 1992). Las transiciones de una fase a otra ocurren de manera ordenada y están reguladas por varias proteínas. El ciclo celular está íntimamente asociado con el crecimiento celular y en consecuencia con el tamaño de la célula, y en levaduras hay dos puntos de control del tamaño distintos. En la levadura de gemación *Saccharomyces cerevisiae* las células deben alcanzar un tamaño crítico antes de replicar su genoma, siendo su principal punto de control la transición G_1-S (Neufeld and Edgar, 1998). En *Schizosaccharomyces pombe*, la levadura de fisión, el crecimiento tiene lugar una vez que el ADN se ha replicado, de manera que su principal punto de control del tamaño es la transición G_2-M (Mitchison and Nurse, 1985). Las proteínas reguladoras clave que controlan las transiciones de una fase a otra son las quinasas dependientes de ciclina (CDKs), que son serina/treonina quinasas que necesitan unirse a una ciclina para estar activas (Pines and Hunter, 1991).

En levaduras hay una única Cdk, llamada Cdc28 en *S. cerevisiae y* Cdc2 en *S. pombe*, las cuales mediante su asociación con diferentes ciclinas regulan el ciclo celular (Nurse and Bissett, 1981; Reed and Wittenberg, 1990). Antes de unirse a una ciclina la Cdk tiene que ser activada por una quinasa de activación de Cdk (CAK) en los residuos Thr169 o Thr167 (*S. cerevisiae* y *S. pombe* respectivamente) (Kaldis, 1999). Las ciclinas se agrupan principalmente en dos grupos, G1 y de tipo B o mitóticas, dependiendo de la transición del ciclo celular que controlen (Pines, 1993). En las transiciones G_1-S, los complejos activos de Cdk/ciclina pueden ser inhibidos directamente por la unión de inhibidores de Cdk como ScSic1 y SpRum1 o indirectamente mediante la degradación dirigida llevada a cabo por los complejos ScCdh1-APC y SpSte9-APC. Durante las transiciones G_2-M los complejos Cdk/ciclina activos pueden ser inhibidos mediante la fosforilación de los residuos Tyr19 o Tyr15 (*S. cerevisiae* y *S. pombe* respectivamente) llevada a cabo por las proteínas tirosina quinasa ScSwe1 o SpWee1 (Rupes, 2002; Russell and Nurse, 1987). La reactivación de los complejos Cdk/ciclina y por tanto la entrada en mitosis la llevan a cabo las proteínas tirosina fosfatasa ScMih1 y SpCdc25 que defosforilan los residuos Tyr fosforilados (King et al., 1994; Russell and Nurse, 1986).

3.1. Regulación del ciclo celular en *U. maydis*

U. maydis comparte similitudes con ambos modelos de levaduras. Al igual que *S. cerevisiae*, es una levadura de gemación aunque la replicación del ADN ocurre antes de que se forme la gema, característica que comparte con *S. pombe* (Snetselaar and McCann, 1997) (Fig. 5). Al contrario que ambas levaduras pero de manera similar a eucariotas superiores, *U. maydis* controla el tamaño celular durante las transiciones G_1-S y G_2-M (Perez-Martin et al., 2006).

U. maydis tiene una CDK llamada Cdk1 que se une a tres ciclinas diferentes, Cln1 que es una ciclina de tipo G1 y Clb1 y Clb2 que son de tipo B (Castillo-Lluva and Perez-Martin, 2005; Garcia-Muse et al., 2003; Garcia-Muse et al., 2004). La transición G_1-S está controlada específicamente por el complejo Clb1-Cdk1. La acumulación de este complejo depende del complejo Cru1-APC, que responde a condiciones nutricionales (Castillo-Lluva et al., 2004). El complejo Cln1-Cdk1 también tiene un papel en el control de la longitud de la fase G_1 así como en el control del tamaño y la morfología de la célula (Castillo-Lluva and Perez-Martin, 2005).Una vez que el ADN se ha replicado, la formación de la gema marca el comienzo de la fase G_2, donde la gema

crecerá hasta alcanzar el tamaño adecuado. El comienzo de la mitosis requiere la presencia de los complejos Clb1-Cdk1 y Clb2-Cdk1, pero únicamente el último de estos es el encargado de controlar la longitud de esta fase: bajos niveles de Clb2 inducen un retraso en la entrada en mitosis produciendo células con gemas largas, mientras que una sobreexpresión de *clb2* resulta en células pequeñas que se dividen por septación (Garcia-Muse et al., 2004). La fuerte regulación de los complejos Clb2-Cdk1 se lleva a cabo a través de la fosforilación inhibitoria de Cdk1 por parte de Wee1, ya que el fenotipo observado después del descenso de los niveles de *wee1* recuerda al observado después de la sobreexpresión de *clb2* y viceversa (Sgarlata and Perez-Martin, 2005b). La eliminación de la fosforilación inhibitoria al comienzo de la mitosis la lleva a cabo la fosfatasa Cdc25, de manera que la sobreexpresión de *cdc25* resulta en una fenocopia de la ablación de *wee1* y viceversa (Sgarlata and Perez-Martin, 2005a). Como en otras levaduras, *U. maydis* tiene una proteína del tipo 14-3-3 llamada Bmh1, que se une a Cdc25 e inhibe su actividad (Mielnichuk and Perez-Martin, 2008). Otros reguladores caracterizados en *U. maydis* que tienen papeles en esta transición celular son Plk1, una proteína de la familia de las poloquinasas y Hsl1 que pertenece a la familia de las proteínas Nim1/Cdr1. Plk1 se ha descrito que tiene un papel en la activación de Cdc25, aunque no se ha podido descartar que ejerza también un posible control sobre la actividad de Wee1. Para Hsl1, se ha propuesto que tiene un papel en el control de la longitud de la G_2 (Mielnichuk, 2007) (Fig. 6).

3.2. Ciclo celular y virulencia en *U. maydis*

La virulencia y el desarrollo sexual están fuertemente unidos en *U. maydis*. Un prerrequisito de ambos procesos es el apareamiento de dos células haploides levaduriformes compatibles para dar lugar a un filamento dicariótico. Este proceso implica cambios morfológicos y genéticos, debido a lo cual es esencial un control preciso del ciclo celular y de la morfogénesis. Condiciones nutricionales pobres como las que se espera que se den en la superficie de la hoja llevan a una estimulación de la expresión de *prf1* (Castillo-Lluva and Perez-Martin, 2005). Prf1 controla la transcripción de los genes *a* y *b* (Hartmann et al., 1996). Los genes *a* codifican un sistema feromona-receptor y activan la formación de tubos conjugativos y la fusión celular (Spellig et al., 1994). Como ocurre en otros hongos, el reconocimiento de la feromona bloquea la progresión del ciclo celular para preparar a las células que se van a aparear para la conjugación, lo que en *U. maydis* se traduce en una parada del ciclo

celular en G_2 (Garcia-Muse et al., 2003). Aparte de esto, el reconocimiento de la feromona activa una cascada de MAPK que lleva a la activación de varios genes entre los que se encuentran los genes *b* (Kaffarnik et al., 2003). La fusión de los tubos conjugativos marca el inicio de la formación de una célula dicariótica con un fuerte crecimiento polar en la cual el ciclo celular permanece parado hasta que el hongo entra dentro de la planta (Snetselaar and Mims, 1994). También, al fusionarse las células, ocurre la interacción entre las dos subunidades que forman el factor b, bW y bE y todos los procesos controlados por las proteínas b se activan. Dentro de estos procesos están el mantenimiento del crecimiento filamentoso y de la parada del ciclo celular.

La parada del ciclo celular en G_2 inducida por el complejo heterodimérico de las b depende de la fosforilación inhibitoria de Cdk1 en el residuo Tyr15. De manera que al inducir la formación del factor b, los niveles de Tyr^{15}P-Cdk1 aumentan fuertemente y la sobreexpresión de una Cdk1 incapaz de ser fosforilada en presencia de las proteínas b lleva a la formación de filamentos multinucleados (Mielnichuk et al., 2009). Wee1 y Cdc25 regulan en la acumulación de Tyr^{15}P-Cdk1. El papel de Wee1 en está parada del ciclo celular asociada con la inducción de un fuerte crecimiento polar se correlaciona con el modelo propuesto por Kellog (2003) , donde las proteínas del tipo Wee1 son propuestas como las encargadas de controlar todo el crecimiento polar que ocurre en la célula. En la regulación de Cdk1 en respuesta a las proteínas b, la expresión de Wee1 aumenta al mismo tiempo que Cdc25 es secuestrada en el citoplasma para de esta forma asegurar la fosforilación de Cdk1 y en consecuencia la parada del ciclo celular. Bmh1 es la proteína que se une a Cdc25 y desencadena su acumulación en el citoplasma. Antes de que se produzca esta unión, Cdc25 tiene que ser fosforilada y Chk1, una quinasa de la ruta de respuesta a daño en el ADN es la encargada de llevar a cabo este paso mediante la creación de un sitio de reconocimiento para Bmh1 (Mielnichuk and Perez-Martin, 2008). Células que carecen de Chk1 y expresan proteínas b compatibles presentan un crecimiento filamentoso con varios núcleos en su interior, lo que indica que la parada de ciclo celular está dañada (Mielnichuk et al., 2009). Está parada del ciclo celular en G_2 no es dependiente únicamente de Chk1, ya que la activación de Chk1 es transitoria, de manera que probablemente existan más componentes que participan en esté proceso que tienen que ser caracterizados (Fig.7).

4. La respuesta al daño en el ADN

Las células sufren constantemente daños en su genoma causados bien por agentes ambientales o por agentes internos, debido a esto la evolución ha envuelto el núcleo de la maquinaria que regula el ciclo celular con mecanismos de vigilancia llamados checkpoints de ciclo celular que aseguran la correcta transferencia del genoma a la siguiente generación. Uno de estos checkpoints es la llamada respuesta de daño en el ADN (DDR), cuyo papel es detectar el ADN dañado y coordinar su reparación con la progresión del ciclo celular (Abraham, 2001; Nyberg et al., 2002). La idea actual es que las rutas de checkpoint de daño en el ADN funcionan en condiciones normales y cuando aumenta el daño en el ADN son amplificadas (Sancar et al., 2004). La ruta de checkpoint de daño en el ADN está formada principalmente por tres grupos de proteínas agrupadas en sensores, transductores de la señal y efectores.

Los sensores son las proteínas que reconocen el ADN dañado y señalizan la presencia de anormalidades. Entre estos sensores se encuentran varios complejos proteicos: el complejo MRN/MRX, formado por Mre11, Rad50 y Nbs1 o Xrs2 dependiendo del organismo, el complejo 9-1-1 que consta de las proteínas Rad9, Hus1 y Rad1 y el hetrodímero Ku que incluye a las proteínas Ku70 y Ku80 (Falck et al., 2005). El complejo MRN participa en el reclutamiento de ATM a las roturas de doble cadena del ADN (DSBs) (Carson et al., 2003). Se une al ADN a través de la proteína Mre11 y dimeriza con otro complejo MRN mediante Rad50 (van den Bosch et al., 2003). El complejo 9-1-1 está asociado con la activación de ATR en respuesta al ADN de cadena sencilla (Navadgi-Patil and Burgers, 2009). Es una molécula heterotrimérica con forma de anillo cuya estructura y secuencia está relacionada con el antígeno nuclear de células proliferativas (PCNA), un anillo móvil replicativo (Parrilla-Castellar et al., 2004). El complejo 9-1-1 reconoce una hebra de ADN adyacente a una región de ADN de cadena sencilla recubierta con RPA (Majka et al., 2006). El hetrodímero formado por Ku70 y Ku80 es el responsable del reclutamiento de la DNA-PKcs a los sitios de DSBs (Gottlieb and Jackson, 1993). Forma una molécula pseudosimétrica con un anillo en su interior con el que rodea al ADN de cadena doble.

Los sensores transmiten la señal a las proteínas quinasa, las cuales transmiten y amplifican la señal de daño mediante la fosforilación de proteínas diana. Las proteínas quinasa que forman este grupo pertenecen a las quinasas relacionadas con la fosfatidilionsitol 3-quinasa (PIKKs) y algunos de los miembros de esta familia son

ATM, ATR y DNA-PKcs. ATM y ATR están bien conservadas entre los eucariotas, mientras que DNA-PKcs solo está presente en vertebrados (Jackson, 2002). Las PIKKs tienen una homología de secuencia significativa entre ellas. Fosforilan residuos de Ser o Thr seguidos por Gln y activan una series de substratos superpuestos que promueven la parada del ciclo celular y la reparación del ADN (Cimprich and Cortez, 2008)

ATM fue identificada en los años 90 como el gen defectuoso en el síndrome AT (ataxia-telangiectasia) que causa desordenes neurodegenerativos y predisposición a cáncer (Savitsky et al., 1995). Las células que carecen de ATM presentan inestábilidad cromosómica y sensibilidad a radiaciones ionizantes y drogas radiomiméticas. Estas células, en presencia de daño inducido por radiación, son defectuosas en los checkpoints del ciclo celular de G_1-S, S y G_2-M (Abraham, 2001). Cuando se forman DSBs, ATM es reclutada a los sitios de daño por el complejo MRN, el cual fosforila ATM a través de su interacción con Nbs1 (Abraham and Tibbetts, 2005).

ATR (relacionada con ATM y Rad3) está relacionada con el síndrome de Seckel, una enfermedad humana rara caracterizada por un retraso en el crecimiento y microcefalia (O'Driscoll et al., 2003). Trastornos en la ruta de ATR producen inestábilidad genómica. ATR se activa en respuesta a la presencia de fragmentos de ADN de cadena sencilla. En la célula, la proteína de replicación A (RPA) cubre a la mayoría del ADN de cadena simple (Fanning et al., 2006), de manera que la estructura que provoca la activación de la ruta de checkpoint dependiente de ATR es RPA unida a ADN de cadena sencilla próxima a una región de ADN de doble cadena que presenta un extremo 5'. Está estructura se genera durante la replicación, en la resección de los extremos durante la reparación de DSBs, en los telómeros y durante la reparación por escisión de nucleótidos (Cimprich and Cortez, 2008). El reconocimiento de RPA cubriendo ADN de cadena simple por parte de ATR depende de la proteína de interacción con ATR (ATRIP) cuya unión es esencial para la actividad de ATR (Ball and Cortez, 2005). Para la activación de ATR en algunos casos se necesita la proteína de unión a la topoisomerasa 1 (TopBP1) (Delacroix et al., 2007). En estos casos las estructuras que producen la activación de ATR también son reconocidas por el complejo 9-1-1 el cual recluta a TopBP1 (Lee et al., 2007). TopBP1 contiene un dominio de activación de ATR que cuando interacciona con ATR lo puede fosforilar.

DNA-PKcs juega un papel critico en la estabilización de los extremos de los DSBs durante el proceso de unión de extremos no homólogos (NHEJ) mediante la prevención de la resección de los mismos y la promoción de la unión de estos. La

interacción con el hetrodímero Ku es a través de un dominio presente en Ku80. Esta interacción estabiliza la unión de la DNA-PKcs al ADN y la activa para que inicie el NHEJ (Ciccia and Elledge, 2010; Durocher and Jackson, 2001).

Finalmente, las quinasas efectoras normalmente regulan dianas que previenen la progresión del ciclo celular mediante la fosforilación de estas. Chk1 y Chk2/Rad53 forman este grupo y son quinasas serina/treonina necesarias para parar el ciclo celular en respuesta a daño en el ADN. Estas quinasas son las diana de ATM y ATR y como tal son fosforiladas de manera dependiente por estas. La fosforilación de Chk1 y Chk2 se produce a través de mediadores, proteínas que simultáneamente asocian transductores con efectores. Dentro de estas proteínas mediadoras se encuentran MDC1, 53BP1, Claspina y BRCA1 junto con sus respectivos homólogos de levaduras (Nyberg et al., 2002; Sancar et al., 2004). La activación de Chk1 y Chk2 desencadena la fosforilación de Cdc25, lo que resulta bien en la creación de un lugar para la unión de 14-3-3 en el caso de Chk1 o bien en una marca para inducir su degradación en el caso de Chk2/Rad53 (Abraham, 2001). De manera que como resultado de esta fosforilación Cdc25 puede ser secuestrada en el citoplasma, degradada o ambas cosas. La inactivación de Cdc25, como hemos visto previamente, lleva a una parada del ciclo celular (Sanchez et al., 1997) (Fig. 8).

4.1. La ruta de checkpoint de daño en el ADN en *U. maydis*

Durante muchos años *U. maydis* ha sido un sistema modelo en el estudio de los procesos de reparación del ADN en respuesta a radiación ultravioleta o ionizante, de hecho, los primeros mutantes eucariotas aislados en reparación del ADN se obtuvieron en *U. maydis* (Holliday, 1965). En los últimos años las rutas de reparación del ADN, especialmente la recombinación homóloga, se han estudiado y caracterizado (Holloman et al., 2008). Curiosamente, el conocimiento de las rutas de señalización de daño en *U. maydis* es muy escaso. Entre los sensores y los transductores de la señal se han encontrado homólogos del complejo MRN, del 9-1-1, del heterodímero Ku, de RPA, TopBP1, ATM y ATR, algunos se han predicho mediante una búsqueda por homología mientras que otros como RPA y Rec1 (homólogo de Rad1) han sido caracterizados (Holliday et al., 1976; Sanchez-Alonso and Guzman, 2008; Thelen et al., 1994; Yang et al., 2005). Pero no se han identificado homólogos para DNA-PKcs, ATRIP o las proteínas mediadoras. Dentro de los efectores, solamente se ha caracterizado Chk1 en respuesta al daño en el ADN ya que *U. maydis* carece de homólogo para Chk2/Rad53.

Chk1 es fosforilada en respuesta a distintos tipos de daño en el ADN y su deleción impide que las células ajusten su ciclo celular a la presencia del daño (Perez-Martin, 2009). Bmh1, la proteína de tipo 14-3-3 en *U. maydis*, participa en la respuesta al daño en el ADN, lo que sugiere una conservación de la ruta de checkpoint de daño en el ADN descrita (Mielnichuk and Perez-Martin, 2008) (Fig. 9).

5. Los telómeros

La organización del genoma en cromosomas lineales requiere la presencia de mecanismos que protejan y aseguren que sus extremos sean completamente duplicados cuando los cromosomas sean replicados. Debido a esto, las células eucariotas han desarrollado los telómeros y la telomerasa. Los telómeros son complejos nucleoproteicos especializados que permiten a la célula distinguir entre extremos cromosómicos y sitios de daño en el ADN. En mamíferos, este complejo proteico se llama complejo protector y en levaduras es conocido como telosoma. La telomerasa es la enzima encargada de alargar las regiones terminales del ADN telomérico (Denchi, 2009).

En eucariotas el ADN telomérico está formado por repeticiones en tándem de secuencias cortas ricas en G, como TTAGGG (Greider, 1996). En lugar de presentar extremos romos, los telómeros terminan en una hebra 3' monocatenaria que invade el ADN telomérico de cadena doble para alinearse con la hebra rica en C complementaria, formando de esta manera una estructura secundaria llamada bucle-t (Griffith et al., 1999). Adyancentemente a estas repeticiones teloméricas terminales se encuentran las secuencias subteloméricas. Estas secuencias también están repetidas en tándem y son similares entre los telómeros aunque difieren mucho entre especies (Blackburn, 2001).

La telomerasa es una enzima ribonucleoproteica que mediante el uso de un molde de ARN añade las secuencias de repeticiones teloméricas en tándem para mantener la longitud de los extremos de los cromosomas, es por lo tanto una enzima que alarga los telómeros de manera especifica. Está formada por una transcriptasa inversa muy conservada que lleva asociada un ARN que sirve como plantilla (Greider and Blackburn, 1989; Nakamura et al., 1997). Normalmente la telomerasa también está formada por proteínas adicionales que no son esenciales para la catalisis *per se*, pero cuya presencia es necesaria para el correcto mantenimiento de los telómeros. Estas proteínas son: dos proteínas accesorias llamadas Est1 y Est3 en *S. cerevisiae* y EstA y EstB en humanos que se unen al ARN de la telomerasa y otra proteína que también

interacciona con el ARN llamada disquerina o proteína Sm (en humanos y *S. cerevisiae* respectivamente) (Smogorzewska and de Lange, 2004).

La composición del complejo protector (en inglés "shelterin") en humanos y del telosoma en levaduras varia, aunque algunos componentes esenciales están conservados en ambas estructuras. Las proteínas que forman ambos complejos se localizan en la región de las repeticiones teloméricas. En ambos complejos hay proteínas del tipo TRF que se unen al ADN de cadena doble, TRF1 y TRF2 en humanos y Taz1 en *S. pombe*, las cuales son esenciales para controlar la longitud del telómero y prevenir las fusiones de estos (Bae and Baumann, 2007; Ferreira and Cooper, 2001).Una característica importante de estas proteínas es que son necesarias para la formación de los bucles-t de los telómeros (Tomaska et al., 2004). También hay otra proteína presente tanto en humanos como en *S. pombe* y que se une al ADN de cadena simple presente en los extremos 3' monocatenarios. Esta proteína se llama Pot1 (del inglés "Protection of telomeres") protege a los telómeros de ser degradados y en humanos regula la actividad de la telomerasa (Baumann and Cech, 2001). Además de estas proteínas, hay otras que completan el complejo de protección de los telómeros o telosoma como: TIN2, TPP1 y Rap1 en humanos o Poz1, Tpz1 y Rap1 en la levadura de fisión (de Lange, 2005; Kanoh and Ishikawa, 2001) (Fig. 10).

A parte del las proteínas que forman el complejo protector, también hay proteínas que no son especificas del telómero pero que transitoriamente se asocian con estos. La mayoría de estas proteínas intervienen en la señalización del daño en el ADN y en procesos de reparación, algunas de ellas son: el complejo MRN, la DNA-PKcs, el heterodímero Ku, Rad3 y Rad26 (homólogos de ATR y ATRIP en la levadura de fisión), ATM, y BLM y WRN, helicasas RecQ y Rad51 estas últimas miembros de la ruta de recombinación homóloga (Palm and de Lange, 2008).

5.1. Los telómeros y la respuesta al daño en el ADN

Un papel esencial de las proteínas asociadas al telómero es el evitar que las rutas de señalización de daño en el ADN reconozcan los telómeros como sitios de ADN dañado. ATM y ATR como transmisoras de estas señales son las proteínas diana cuya activación es suprimida.

En humanos, la deleción de TRF2 lleva a la activación de la respuesta de daño en el ADN mediante la ruta del complejo MRN-ATM (Deng et al., 2009). De manera que se ha propuesto que la inhibición de ATM en los telómeros está controlada por

TRF2, la cual se puede unir a ATM e inhibir su fosforilación y en consecuencia la activación de la ruta de respuesta a daño en el ADN en condiciones normales (Karlseder et al., 2004). El mecanismo de activación de ATR depende del reclutamiento de RPA a los extremos monocatenarios ricos en G. En los telómeros RPA compite con POT1 por el ADN de cadena sencilla, competición que en condiciones normales gana Pot1 debido a que pertenece al complejo protector (Denchi and de Lange, 2007) (Fig. 11).

En *S. pombe*, el complejo 9-1-1, Rad3 y Rad26 (homólogos de ATR y ATRIP respectivamente) están unidos a los telómeros y son importantes para el mantenimiento de estos (Nakamura et al., 2002). La inhibición de la respuesta al daño en el ADN que finalmente lleva a la fosforilación de Chk1 seguida de la parada del ciclo celular, está producida por Pot1, Ccq1, que es una proteína que interacciona con Pot1 y necesaria para el reclutamiento de la telomerasa y Taz1. Estas proteínas crean una región privilegiada en la cromatina cromosómica que bloquea la transducción de una señal activa de checkpoint mediante la prevención de la asociación estable del homólogo de 53BP1, Crb2, con los telómeros (Carneiro et al., 2010). Cómo se inhibe Tel1, el homólogo de ATM en la levadura de fisión, de momento no se sabe.

5.2. Los telómeros y la reparación del ADN

El complejo protector no solo suprime la activación de la respuesta al daño en el ADN sino que también es necesario para reprimir reacciones inapropiadas de reparación en los extremos de los cromosomas. La reparación por escisión de bases (BER) y de emparejamientos erróneos (MMR) se usan en el mantenimiento de la secuencia de repeticiones TTAGGG, mientras que la activación de otros mecanismos de reparación como el NHEJ o la HR podrían tener resultados desastrosos en los telómeros (de Lange, 2005). La activación del NHEJ resultaría en fusiones de los extremos de los cromosomas dando lugar a cromosomas dicéntricos que no podrían segregarse bien en mitosis. TRF2, componente del complejo protector interviene en la supresión de la ruta NHEJ. En ausencia de TRF2, la formación de los bucles-t no se puede producir correctamente y los extremos monocatenarios 3’ son eliminados por una exonucleasa. Esto lleva a la formación de DSBs y a la unión del heterodímero Ku junto con la DNA-PKcs desencadenando la reparación de los extremos mediante la actividad de la Ligasa IV (Denchi, 2009). Un miembro interesante de está ruta es el heterodímero Ku cuya actividad todavía tiene que esclarecerse. Las proteínas Ku parece que tienen papeles contradictorios en los telómeros, por un lado intervienen en la ruta NHEJ y al mismo

tiempo se han descrito como protectoras de los telómeros para evitar las fusiones de los extremos (Fisher and Zakian, 2005). La interpretación actual de esta paradoja es que el complejo protector de los telómeros modularía la actividad del heterodímero Ku (de Lange, 2009). La activación del otro mecanismo de reparación del ADN, la recombinación homóloga, en los telómeros generaría telómeros de longitud aberrante, deleciones teloméricas y translocaciones. TRF2 también participa en la supresión de la ruta de recombinación homóloga en los telómeros. Aún más, TRF2 y Ku70 parece que actúan de manera redundante en la prevención de la recombinación en los telómeros (Baumann and Cech, 2000) (Fig. 12).

5.3. Los telómeros en *U. maydis*

La información sobre la organización de los telómeros en *U. maydis* es muy escasa. Las repeticiones teloméricas han sido identificadas y su secuencia, TTAGGG, es la misma que en la mayoría de los eucariotas. Adyacentemente a las repeticiones teloméricas, se encuentran dos secuencias repetitivas presentes en varios de los extremos de los cromosomas que tienen longitud polimórfica. Estas secuencias se conocen como secuencias asociadas a los telómeros de *Ustilago maydis* o *UTAS*. *UTASa* está muy conservada y se localiza principalmente en los extremos de los cromosomas, mientras que *UTASb* está menos conservada y se puede encontrar por todo el genoma (Sanchez-Alonso and Guzman, 1998). Estos son todos los datos experimentales disponibles por el momento sobre los telómeros de *U. maydis*. El resto es solo una predicción de los componentes de la telomerasa y el complejo protector hecha por homología de secuencia a través de la base de datos del genoma anotado de *U. maydis*. Entre estos datos, los componentes de la telomerasa que se han identificado son la unidad catalítica de la transcriptasa inversa, la disquerina y Est1. Al contrario que la identificación de los componentes de la telomerasa, del complejo protector solo se han identificado los componentes que se unen al ADN: Pot1 y Trf1 (Sanchez-Alonso and Guzman, 2008).

Objectives

The objectives of this study were the following ones:

1. Characterization of the upstream activators of Chk1 in DNA damage checkpoint pathway in *Ustilago maydis*.

2. Role of the DNA damage checkpoint pathway during the pathogenic stage of *U. maydis*.

3. Study different stimuli that modulate the Atr1-Chk1 pathway in *U. maydis*.

Materials & Methods

1. Strains and growth conditions

1.1. Strains and plasmids

U. maydis strains used in this work as well as their genotypes and origins are listed in table 1. *Coprinopsis cinerea* strains are included in table 2. Plasmids used in the different genetic constructs are in table 3. For cloning purposes *Escherichia coli* DH5α strain was used.

Table 1: *U. maydis* strains used in this study

Strains	Genotype	Origin	Reference
FB1	*a1b1*	521x518	Banuett & Herskowitz, 1989
FB2	*a2b2*	521x518	Banuett & Herskowitz, 1989
UCM350	*a1b1 pan1-1 nar1-6*		Holliday, 1991
UCM520	*a2b2 met1-2 nar1-1*		Kojic et al., 2002
UCS1	*a1b1 Δatr1*	FB1	This work
UCS6	*a2b2 Δatr1*	FB2	This work
UCS4	*a1b1 a2b2 Δatm1*	FBD11	This work
UCS9	*a1b1 pan1-1 nar1-6 Δatr1*	UCM350	de Sena-Tomás et al., 2011
UCS10	*a2b2 met1-2 nar1-1 Δatr1*	UCM520	de Sena-Tomás et al., 2011
UMP122	*a1b1 Δchk1*	FB1	Pérez-Martín, 2009
UMP129	*a2b2 Δchk1*	FB2	Mielnichuk et al., 2009
UCM565	*a1b1 pan1-1 nar1-6 Δbrh2*	UCM350	Kojic et al., 2002
UCM575	*a2b2 met1-2 nar1-1 Δbrh2*	UCM520	Kojic et al., 2002
UCS22	*a2b2 Δatr1 Δchk1*	UCS1xUMP129	de Sena-Tomás et al., 2011
UCS27	*a1b1 pan1-1 nar1-6 Δbrh2 Δatr1*	UCM350	de Sena-Tomás et al., 2011
AB5	*a1 P_{nar1}:bW2 P_{nar1}:bE1*	FB1	Brachmann et al., 2001
AB33	*a2 P_{nar1}:bW2 P_{nar1}:bE1*	FB2	Brachmann et al., 2001
UMP114	*a2 P_{nar1}:bW2 P_{nar1}:bE1 Δchk1*	AB33	Mielnichuk et al., 2009
UCS31	*a1 P_{nar1}:bW2 P_{nar1}:bE1 P_{tef}:T7-chk1*	AB5	de Sena-Tomás et al., 2011
UCS32	*a1 P_{nar1}:bW2 P_{nar1}:bE1Δatr1 P_{tef}:T7-chk1*	UCS17	de Sena-Tomás et al., 2011
UMP162	*a1b1 P_{tef}:T7-chk1*	FB1	de Sena-Tomás et al., 2011
UMP207	*a1b1 P_{tef}:T7-chk1 Δatr1*	UCS1	de Sena-Tomás et al., 2011
UMP111	*a1b1 chk1-3GFP*	FB1	Pérez-Martín, 2009

UCS15	*a1b1 Δatr1 chk1-3GFP*	UCS1	de Sena-Tomás et al., 2011
UCS20	*a1 P_{nar1}:bW2 P_{nar1}:bE1 P_{dik6}:NLS-3GFP*	AB5	de Sena-Tomás et al., 2011
UMP112	*a2 P_{nar1}:bW2 P_{nar1}:bE1 P_{dik6}:NLS-3GFP Δchk1*	UMP114	Mielnichuk et al., 2009
UCS21	*a1 P_{nar1}:bW2 P_{nar1}:bE1 P_{dik6}:NLS-3GFP Δatr1*	UCS20	de Sena-Tomás et al., 2011
UMP196	*a1b1 P_{tef}:NLS-3GFP*	FB1	de Sena-Tomás et al., 2011
UMP197	*a1b1 P_{tef}:NLS-3GFP Δbrh2*	UMP196	de Sena-Tomás et al., 2011
UMP198	*a1b1 P_{tef}:NLS-3GFP Δatr1*	UMP196	de Sena-Tomás et al., 2011
UMP199	*a1b1 P_{tef}:NLS-3GFP Δchk1*	UMP196	de Sena-Tomás et al., 2011
UMP133	*a2 P_{nar1}:bW2 P_{nar1}:bE1 chk1-3GFP*	AB33	Mielnichuk et al., 2009
UMP208	*a2 P_{nar1}:bW2 P_{nar1}:bE1 chk1-3GFP Δatr1*	UCS17	de Sena-Tomás et al., 2011
UMP183	*a2 P_{nar1}:bW2 P_{nar1}:bE1 P_{dik6}:NLS-3GFP $chk1^{T394A\ S448A}$-T7*	UCS20	de Sena-Tomás et al., 2011
UMP190	*a1b1 $chk1^{T394A\ S448A}$-T7*	FB1	de Sena-Tomás et al., 2011
UMP191	*a2b2 $chk1^{T394A\ S448A}$-T7*	FB2	de Sena-Tomás et al., 2011
UMP168	*a2 P_{nar1}:bW2 P_{nar1}:bE1 P_{gap1}:GFP-rad51*	AB33	Mielnichuk et al., 2009
UCS33	*a1b1 P_{nar1}:uku70*	FB1	This work
UCS34	*a1b1 P_{nar1}:uku70 cut11-Cherry*	UCS33	This work
UCS42	*a1b1 P_{nar1}:uku70 chk1-3GFP*	UCS33	This work
UCS35	*a1b1 P_{nar1}:uku70 Δchk1*	UCS33	This work
UCS40	*a1b1 P_{nar1}:uku70 Δatr1*	UCS33	This work
UCS50	*a1b1 P_{nar1}:uku70 Δchk1 Δbrh2*	UCS35	This work
UCS56	*a1b1 P_{nar1}:uku70 Δbrh2*	UCS55	This work
UCS45	*a1b1 P_{nar1}:uku70 pot1-Cherry P_{gap1}:GFP-rad51*	UCS33	This work
UCS48	*a1b1 P_{nar1}:uku70 pot1-Cherry P_{gap1}:GFP-rad51 Δchk1*	UCS45	This work
UCS51	*a1b1 P_{nar1}:uku70 pot1-Cherry*	UCS45	This work

	P_{gap1}:GFP-rad51 Δatr1		
UCS30	*a1b1 P_{nar1}:uku80*	FB1	This work
UCS37	*a1b1 P_{nar1}:uku80 cut11-Cherry*	UCS30	This work
UCS43	*a1b1 P_{nar1}:uku80 chk1-3GFP*	UCS30	This work
UCS39	*a1b1 P_{nar1}:uku80 Δchk1*	UCS30	This work
UCS44	*a1b1 P_{nar1}:uku80 Δatr1*	UCS30	This work
UCS46	*a1b1 P_{nar1}:uku80 pot1-Cherry P_{gap1}:GFP-rad51*	UCS30	This work
UCS49	*a1b1 P_{nar1}:uku80 pot1-Cherry P_{gap1}:GFP-rad51 Δchk1*	UCS46	This work
UCS52	*a1b1 P_{nar1}:uku80 pot1-Cherry P_{gap1}:GFP-rad51 Δatr1*	UCS46	This work
UCS57	*a1b1 pot1-3GFP P_{gap1}:YFP-rad51*	UMP192	This work

Table 2: *C. cinerea* strains used in this work

Strain	Genotype	Origin	Reference
AmutBmut	*A43mut B43mut pab-1*		Swamy et al., 1984
CCS1	*A43mut B43mut pab-1* RNAi control	AmutBmut	This work
CCS2	*A43mut B43mut pab-1* RNAi *chk1*#1	AmutBmut	This work
CCS3	*A43mut B43mut pab-1* RNAi *chk1*#2	AmutBmut	This work
CCS4	*A43mut B43mut pab-1* RNAi *atr1*#1	AmutBmut	This work
CCS5	*A43mut B43mut pab-1* RNAi *atr1*#2	AmutBmut	This work

Table 3: Plasmids used in this study

Plasmid	Reference
pUMA261	Brachmann et al., 2004
pUMA263	Brachmann et al., 2004
pRU2	Brachmann et al., 2004
pCU3-T7-chk1	Pérez-Martín, 2009
pchk1-3GFP	Pérez-Martín, 2009
pDik6-NLSGFP	Mielnichuk et al., 2009
pCU3-NLS-3GFP	
pCut11-Cherry	Pérez-Martín, 2009
pPot1-3GFP	Laboratory collection

pRad51-GFP	Kojic et al., 2005
pPot1-cherry Pgap1:3GFP-rad51	Laboratory collection
pGEM-T-easy	Promega
pYSK7	Kilaru et al., 2006
pGH-pab	This work

1.2. Growth media and conditions

1.2.1. General media and conditions

For cloning purposes *E. coli* was grown at 37ºC in LB (Luria Broth) medium (Sambrook et al., 1989) supplemented with ampicillin at 100μg/ml.

U. maydis strains were grown in yeast peptone (YP) (Kaiser et al., 1994) or complete medium (Holliday, 1974a). Minimal medium with nitrate ($MMNO_3$) or ammonium ($MMNH_4$) were prepared according to Alfa et al. (1993). As carbon source, glucose was added to all media in a 1% final concentration. In solid media, agar at 2% was used. For the mating experiments, charcoal was added to the media at 1% final concentration (Holliday, 1974b). When needed, media were supplemented with antibiotics: Hygromycin B (200μl/ml), Carboxin (2μl/ml), Nourseothricin (150μl/ml) or Phleomycin (5μl/ml). *U. maydis* strains were grown at 28ºC, and liquid cultures were shaken at 250rpm.

C. cinerea strains were grown in yeast malt extract with glucose (YMG) (Rao and Niederpruem, 1969) and minimal medium (MM) (Granado et al., 1997). Plates were grown at 37ºC in dark and in a humid environment.

All chemicals used were of analytical grade and were obtained from Sigma, Merk or Difco. All the used media were prepared with Milli-Rho water.

1.2.2. Regeneration conditions and selection of transformants

U. maydis protoplasts were grown in regeneration agar (Schulz et al., 1990) prepared with yeast extract, bacto peptone, sorbitol and sucrose as carbon source. Regeneration plates were made with two different agar layers, the first one with two times the antibiotic concentration described above and the second one without antibiotic.

C. cinerea protoplasts were grown in regeneration medium as described by Granado et al. (1997).

1.2.3. Serial dilutions and genotoxic sensitivity analysis

For serial dilutions, 1ml of culture grown to O.D = 0,8 – 1 was diluted 1:2 and from this dilution 4 serial 1:10 dilutions were made. These dilutions were plated in YP or $MMNO_3$ supplemented with 0,5mM Hydroxyurea (HU), 0,01% Methyl methanesulfonate (MMS) or 10ng/ml Phleomycin (Phleo). To test the sensitivity to UV radiation and to γ-radiation, plates were treated with different doses: $50J/m^2$, $150J/m^2$ and $300J/m^2$ or 50Gy, 150Gy and 300Gy respectively. The plates were incubated 2 days at 28ºC

To check *C. cinerea* sensitivity to different genotoxic stresses a cylindrical piece of mycelium with a diameter of 4mm was inoculated on YMG plates supplemented with 2,5mM HU or 0,015% MMS. Plates were grown at 37ºC in a dark humid environment until the plates without genotoxic agents were fully covered of mycelium.

1.2.4. Inducible and constitutive promoters in *U. maydis* and *C. cinerea*

In *U. maydis* experiments where a controlled expression of the genes was required, the inducible promoter P*nar1* (from the nitrate reductase gene) was used, which is activated in presence of nitrate and is repressed with ammonium (Brachmann et al., 2001). Cells were grown in $MMNO_3$ until they reached an O.D = 0,6. Then they were washed 3 times with sterile water and finally were transferred to a repressive medium. Another inducible promoter used was P*dik6*, which is activated in the presence of the bW-bE heterodimer (Flor-Parra et al., 2006).

When a constitutive expression of the genes was needed, the P*tef*, promoter of the translation elongation factor 2 gene or the P*gap*, from the glyceraldehyde-3-phosphate dehydrogenase gene were used (Smith and Leong, 1990; Spellig et al., 1996). Cells were grown until an O.D = 0,6 – 0,8 was reached and then the experiment was performed.

In *C. cinerea*, *Agaricus bisporus* strong constitutive promoter P*gpdII*, from the glyceraldehyde-3-phosphate dehydrogenase gene was used (Kilaru et al., 2006).

2. Genetic methodology

2.1. Cloning and restriction mapping

E. coli plasmid DNA extraction, DNA restriction enzyme digestions, alkaline-phosphatase treatment and electrophoretic analysis were performed as described by Ausubel et al. (1997). Restriction enzymes were provided by New England Biolabs. Ligations were carried out using T4 DNA Ligase from Roche. DNA fragment isolation from TAE electrophoresis gels was done according to QIAGEN QIAquick Gel Extraction Kit. *E. coli* competent cells were transformed with purified plasmids or ligation mixes by the heat shock method (Hanahan, 1983).

2.2. PCR reaction

PCR amplification was performed in a thermocycler machine. Mix and PCR program were adjusted in function of the DNA polymerase used. The elongation time was set according to the size of the amplified fragment and the annealing temperature according to the oligonucleotides used. DNA polymerases used in this study were: Taq (own production), Expand Long Template (from Roche) and Pwo (from Roche). Reaction mix had 500nM of each oligonucleotide, 10ng DNA, 200µM of each dNTP, 5µl of 10x buffer and one unit of polymerase per 50µl of reaction volume. In high fidelity amplifications Pwo and Expand Long Template polymerases were used whereas in the rest of the amplifications Taq was used.

2.3. Genomic DNA extraction from *U. maydis*

U. maydis genomic DNA extraction was performed as described by Hoffman and Winston (1987). According to this protocol, cells were mechanically lysed. Firstly, cells were grown overnight until they reached an O.D = 0,8 – 1 and then 1,5ml of the culture was spun down at 14000rpm for 2 minutes. After discarding the supernatant 500µl of lysis buffer (10mM Tris-HCl pH 8, 1mM EDTA, 100mM NaCl, 1% SDS and 2% Triton) were added together with glass beads (Sigma). Cells were smashed in a Hybaid ribolyser set at speed 6 for 20". After lysis, a phenol: chloroform DNA extraction was done. Finally, DNA was resuspended in 50µl of distilled water by shaking the samples at 70ºC for 5 minutes.

2.4. *U. maydis* transformation

U. maydis transformation was performed according to the protocol (Schulz et al., 1990). In summary, log-phase cells were incubated with Novozym328 in SCS buffer (1M sorbitol, 20mM sodium citrate pH 5,8) until they became round. Then they were washed and resuspended in STC buffer (1M sorbitol, 10mM Tris-HCl pH 7,5, $CaCl_2$) and were kept at -80ºC until they were used. All the solutions were prepared in Milli-Q water. The transformation was done on ice by first incubating the protoplasts with a mix of heparin and linear DNA and then by adding a STC/40%PEG3350 solution. Finally, protoplasts were spread on freshly prepared regeneration agar plates and grown at 28ºC.

2.5. Genomic DNA extraction from *C. cinerea*

C. cinerea genomic DNA extraction was performed according to the protocol described by Zolan and Pukkila (1986). Briefly, fully covered plates with mycelium were ground in liquid nitrogen and then 600µl of DNA extraction buffer (1% CTAB, 0,7M NaCl, 50mM Tris pH 8, 10mM EDTA, 1% 2-mercaptoethanol) were added. The tubes were vortexed and incubated at 60ºC for 30 minutes. Then two chloroform: isoamylalcohol (24:1) DNA extractions were made. After precipitating the DNA with ethanol and drying the pellet, the DNA was resuspended in 50µl of distilled water.

2.6. *C. cinerea* transformation

C. cinerea oidia were transformed as described by Granado et al. (1997). Oidia were harvested and incubated in MM buffer (0,5M mannitol, 50mM maleate pH 5,5) containing a mix of Onozuka R10 Cellulase and Chitinase (Sigma C-6137) for several hours at 37ºC until the spores became round. Then they were washed with MM buffer and finally were resuspended in MMC buffer (0,5M mannitol, 50mM maleate pH 5,5, 25mM $CaCl_2$) with 12,5% PEG3350. Protoplasts were stored at -80ºC until they were needed. For the transformation, the protoplasts were firstly incubated on ice with the DNA and then a 25% PEG3350 solution and STC buffer (1M sorbitol, 10mM Tris, 25mM $CaCl_2$) were added before plating them on regeneration medium. Plates were incubated at 37ºC on a dark humid environment.

3. Telomere analysis

3.1. Genomic DNA extraction from *U. maydis*

U. maydis strains were grown on $MMNO_3$ until an O.D = 0,8 – 1 was reached. Subsequently, the cells were washed several times with distilled water before reinoculate them into 800ml of YPD media to let them grow overnight until an O.D = 0,8 – 1. Every 200ml of culture were treated with 100ml of 0,2% azide solution in MilliQ water and after centrifuging and washing the cells with cold MilliQ water protoplasts were done with Novozym as described before. After the cells became round they were washed once with SCS buffer and once with the Inactivation buffer (50mM Tris pH 8.0, 50mM EDTA, 100mM NaCl, 1M Sorbitol). Then the protoplasts were resuspended in NIB buffer (17% glycerol, 50mM MOPS, 150mM K acetate, 2mM $MgCl_2$, 500μM spermidine, 150μM spermine) to break the cells and isolate the nuclei. After this the genomic DNA was extracted with the QIAGEN Genomic-Tip 100/G from the QIAGEN Genomic DNA Extraction Kit and precipitated and washed with isopropanol and 70% ethanol respectively. Finally the DNA was resuspended in 1xTE.

3.2. Southern blotting and hybridization

For the 2D gels, in the first dimension a 0,4% TBE 1x agarose gel was made using Seakem LE agarose (Lonza), where 4μg of well digested genomic DNA were loaded and the gel was run for 20 hours at 0,7 volts/cm (between electrodes). In the second dimension, 10cm long slices from the first gel that contained the bands of interest were included on a 1% TBE 1x agarose gel with EtBr 0,3μg/ml. This gel was run at 4ºC on pre-cooled 1xTBE at 5 volts/cm for 4 hours.

For the 1D gel, a 0,8% TAE 1x agarose gel was made, were 1μg of digested genomic DNA was loaded and was run for 4 hours on 1xTAE at 100 volts.

Both types of gels were transferred to a Hybond-XL (GE Healthcare) membrane, after being depurinated in 0,25N HCl and denatured in a 0,5M NaOH, 1,5M NaCl solution, by a vacuum system at 50mbar.

2D gels were hybridized with an oligo marked with [γ-^{32}P] ATP (Perkin Elmer 6000Ci/mmol). In the oligo labelling, T4 PNK (New England Biolabs) was used. 1D gels were hybridized with a [α-^{32}P] dCTP (Perkin Elmer 6000Ci/mmol) labelled probe. The probe was amplified from genomic DNA with the oligos subtelomeric-1 and

subtelomeric-2. In the labelling process Klenow (from Roche) was used. Prehybridization and hybridization were done in 1% SDS, 1M NaCl, 10% Dextran sulphate, 100μg/ml ssDNA solution at 65ºC for up to 24 hours each. After this the membrane was washed twice with different solutions: 2x SSC at room temperature, 2xSSC, 1%SDS at 65ºC and once with 0,1x SSC at room temperature. Then the blot was exposed to a phosphorimager screen. Oligos are detailed in table 5.

4. Gene expression analysis methods

4.1. RNA extraction from *U. maydis*

Total RNA extraction was carried out according to the method described by Schmitt et al. (1990). Cells were lysated mechanically on TES tampon (10mM Tris-HCl pH 7,5, 10mM EDTA, 0,5% SDS) and RNA was extracted with an acidic phenol solution followed by a chloroform: isoamyl alcohol (24:1) solution. After precipitation with 3M AcNa, RNA was air dried and dissolved in 50-100μl DEPC water. After the extraction, the RNA was cleaned with the High Pure RNA Isolation Kit from Roche where a DNAseI treatment was performed.

4.2. RNA extraction from *C. cinerea*

The RNA extraction from *C. cinerea* was done as described (Chomczynski and Sacchi, 1987). Shortly, plates fully covered of mycelium were ground in liquid nitrogen and 500μl of denaturing solution (4M guanidine thiocyanate, 25mM sodium citrate pH 7, 0,5% N-lauroylsarcosine) were added per sample tube. Then, 2M sodium acetate, acidic phenol and chloroform: isoamyl alcohol (49:1) solutions were added and mixed by vortex. After 15 minutes of ice incubation, the samples were centrifuged at 14000rpm for 10 minutes and the supernatant was precipitated with 2-propanol at -20ºC. After washing the pellet with ethanol and drying the pellet, the RNA was resuspended on DEPC water by shaking at 55ºC 10 minutes. After the extraction, the RNA was cleaned as described before.

4.3. cDNA synthesis

After quantifying the RNA content of the samples with a Nanodrop ND-1000 Spectrophotometer the cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacture's instructions.

4.4. RT-PCR

The cDNA synthesized was diluted 1:10 before being used for the RT-PCR. The reaction mix had 200nM of each oligonucleotide and the *Power* SYBR® Green PCR Master Mix (Applied Biosystems). The machine used was the 7300 Real Time PCR System from Applied Biosystems as well as the software used for the data acquisition. The mathematical equation described by Pfaffl et al., (2004) was used to analyzed the differential gene expression. The efficiencies of the designed oligos, which are listed on table 5, were the following ones:

Table 4: Efficiencies of the oligos used in the quantitative RT-PCR.

Organism	oligos	Efficiency (%)
C. cinerea	benA RT-1 + benA RT-2	94,3
C. cinerea	atr1 RT-5 + atr1 RT-6	96
C. cinerea	chk1 RT-3 + chk1 RT-4	102,2
U. maydis	tub RT-1 + tub RT-2	103,9
U. maydis	ku70 RT-1 + ku70 RT-2	91,5
U. maydis	ku80 RT-1 + ku80 RT-2	97,9

5. Protein analysis methods

5.1. Protein extraction

U. maydis protein extraction was done by lysing the cells mechanically with glass beads on BF buffer (50mM Tris-HCl pH 7,5, 250mM NaCl, 0,1% Triton X100, 50mM NaF, 1mM β-glycerolphosphate, 1mM EGTA, 12,5mM sodium pyrophosphate, 0,1mM $NaVO_3$, 5mM EDTA pH 8, 1mM PMSF and 1/10 of Protease Inhibitor Cocktail Tablets from Roche). The lysates were subsequently used in the inmunoprecipitation process before loading them on gel.

5.2. Inmunoprecipitation

Inmunoprecipitation was carried out with the commercial system Dynabeads® (Invitrogen) according to manufacture's recommendations. The protein extract was incubated with the buffer described above for 2 hours. Then magnetic beads were added (G protein resin bound to magnetic beads) and this mix was incubated 1 hour at 4ºC with slow agitation. Then, the beads were washed 3 times with the same buffer before releasing the proteins from the beads with the lysis buffer (125mM Tris-HCl pH 6,8, 1% β-mercaptoethanol, 4% SDS, 0,005% bromophenol blue, 20% glycerol, 5mM EDTA pH 8, 1mM PMSF and 1/10 of Protease Inhibitor Cocktail tablets). After, the samples were either incubated 5min at 100ºC and loaded on the gel or stored at -80ºC.

5.3. Western blotting

Protein extracts were separated on 8% acrylamide/0,1% bisacrylamide, pH 9,2 gels (Perez-Martin, 2009) and run on Tris-HCl/Glycine/SDS (50mM/400mM/0,02%) with constant amperage. The proteins were transferred to an Inmobilon-P membrane (Millipore) using a Bio-Rad Mini Trans-blot® Cell. The transfer was done at 0,15mA for 25 min on transfer buffer (48mM Tris-HCl pH 7,5, 39mM glycine, 0,0375% SDS, 20% methanol). Blocking was done for 1 hour with 5% milk in PBS and then the membrane was incubated on milk with the antibody for another hour. After this, the membrane was washed with 0,05% Tween in PBS. The Western Lightning Plus Chemiluminiscence Reagent Kit (Perkin Elmer) was used to develop the membranes. The α-[T7-peroxidase] (1:10000 Novogen) was the conjugated antibody used in this study.

6. Genetic screening

uku70^{nar1} strain was grown on $MMNO_3$ overnight at 28ºC until it reached an O.D= 0,8 – 1. After, it was washed extensively with YPD before being grown on this media for 6 hours at 28ºC. Then 100µl of a 1:100 dilution were plated on YPD plates (a total of 20 YPD plates were used) and these were treated with 600J/m^2 of UV light before incubating them at 28ºC. Single colonies were isolated and streaked on YPD and YPD + 0,5mM HU. Those colonies that could grow on YPD but were not able to do so on HU plates were selected. The selected colonies were firstly crossed with compatible strains available in the lab that were defective in the DNA damage checkpoint pathway

(i.e. *Δatr1*, *Δchk1*…). The crosses were done in CMD + charcoal plates and from the mating, diploids were isolated selectively with antibiotics. These diploids were checked for complementation by their sensitivity to HU. Those colonies whose mutation could not be identified by this process were transformed with a *U. maydis* genomic DNA library. All the colonies obtained from this transformation were checked on HU, and from those that had become HU resistant, the plasmid that they carried was isolated, amplified in *E. coli* and sequenced to identify the gene they coded for.

7. Fluorescence-activated cell sorting (FACS)

Analysis of the cellular DNA content by flow cytometry was done according to the protocol described by Garrido and Pérez-Martín (2003). Briefly, cells were fixed with 70% ethanol and then were treated with 0,025mg/ml RNAse A and 1mg/ml Proteinase K. Finally the samples were incubated with 16μg/ml propidium iodide to stain the DNA and were subsequently analyzed with a Coulter XL machine.

8. Microscopy

Samples were visualized in a Nikon Eclipse 90i microscope equipped with a Hamamatsu ORCA-ER CCD camera. Pictures were taken using the appropriate filter set, Nikon Plan Apo *VC* 100X NA 1,40 and Plan Apo *VC* 60X NA 1,40 lenses with Nikon Immersion Oil type A nd=1,5151. The software used with the microscope was Metamorph 6.1 and the pictures were further processed with Adobe Photoshop CS5.

8.1. Nuclear observation

U. maydis nuclear observation was done with strains that carried a *NLS-GFP* sequence under the *dik6* promoter (Mielnichuk et al., 2009) or by staining the nucleus with a DAPI (4',6'-diamidino-2-phenylindole) solution prepared on PBS (Garcia-Muse et al., 2003). Samples were prepared by air-drying 2μl of the culture on the microscope slide and later adding 1μl from a 1μg/ml DAPI solution.

For the observation of the nuclear membrane a *cut11-Cherry* endogenous fusion was introduced in the cells (Perez-Martin, 2009). And to observe Rad51 foci formation after DNA damage a *GFP-rad51* or *YFP-rad51* allele under the constitutive promoter P*gap* was used (Kojic et al., 2005).

8.2. Telomere observation

Telomeres were observed by using an endogenous protein fusion PotI-Cherry or PotI-GFP, depending on the case.

8.3. Septa and nuclei staining in *C. cinerea*

To perform septa and nuclei staining, *C. cinerea* was grown for two days at 37ºC on a thin agar layer over a glass microscope slide with a small window in the middle of the size of the cover slip. After this time, 70% EtOH was added to dry out the hyphae and then a 0,5% DMSO, 140nM Hoechst 33258 solution was added and kept in dark for 15min before observing it.

8.4. *U. maydis* staining *in planta*

Two different methods were used to stain *U. maydis*. For the Chlorazole Black E staining method, 16 days post-infection maize plants were analyzed as described by Brachmann et al.(2003). To visualize fungal hyphae in plant, 2-3 days post-infection maize plants were stained with WGA-AF488 and Propidium Iodide as described by de Sena-Tomás et al.(2011). These samples were analyzed by using a Deltavision wide-field microscope (Applied Precision).

9. *Zea mays* infection

For the pathogenicity assays, 14-days-old maize plants var. Gaspar Flint were infected with 0,5ml of a compatible strains cell suspension of 10^7 cells/ml by using a syringe. The compatible strains had been grown overnight and washed twice with sterile water before the infection. Corn seedlings were grown on a greenhouse and the disease symptoms were evaluated according to the disease characteristic symptoms described by Banuett and Herskowitz (1996).

10. Teliospores germination

Teliospores isolated from *U. maydis* tumours were washed with 1,5% $CuSO_4$ during 24 hours at room temperature with soft rotation. After this, they were washed 6 times with sterile distilled water and finally were sown on plates with the required medium.

11. Fruiting body formation

Induction of fruiting body formation in *C. cinerea* was done by changing the growth conditions. Almost fully covered plates of mycelium grown in dark at 37ºC were incubated on a climate chamber at 30ºC, with 12 hours of light daily and over 85% of humidity (Navarro-Gonzalez, 2008).

12. *In silico* analysis

The homology search of the *U. maydis* Atr1, Atm1, Ku70 and Ku80 proteins was performed with the BLAST program from the *U. maydis* database website (http://mips.helmholtz-muenchen.de/genre/proj/ustilago) using the sequences from *S. cerevisiae* or *S. pombe* as probes. To search for the Atr1 and Chk1 homologues in *C. cinerea*, a BLAST analysis in the *C. cinerea* database webpage (http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/MultiHome.html) was performed using the *U. maydis* sequences as probes. To verify the results a BLAST analysis on the NCBI website (http://www.ncbi.nlm.nih.gov/) using *U. maydis* or *C. cinerea* retrieved sequences, respectively, as probes was done. Domain analysis was done using Pfam database (http://pfam.sanger.ac.uk/) and the Conserved Domain Database from the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

13. *Ustilago maydis* strains constructs

To generate the different strains, the constructs detailed below were used to transform protoplasts. The integration of the plasmids into the corresponding loci by homologous recombination was verified by diagnostic PCR. Oligonucleotides used to perform the constructs appear on the figures in black and those used to check the correct insertion in red. Oligos are detailed in table 5.

13.1. *atr1* deletion construct

Deletion of the *atr1* gene was performed according to the method described by Brachmann et al.(2004). Two PCR fragments flanking the *atr1* open reading frame, ORF, were amplified with atr1-22 and atr1-3 (for the 5' homologous fragment) and with atr1-4 and atr1-23 (for the 3' homologous fragment). Subsequently, these fragments were digested with the restriction enzyme *Sfi*I to be ligated to the carboxin or

hygromycin B resistant gene digested with the same enzyme (from the pUMA260 or pUMA261 plasmid respectively). The obtained cassette was transformed and the transformants were checked by PCR by using the oligos atr1-21 and smut3 (2,1 kb fragment) and atr1-24 and smut4 (2,2 kb fragment) for the hygromycin resistant cassette, and with atr1-21 and cbx1 (2,2kb fragment) and atr1-24 and cbx2 (2,5kb fragment) for the carboxin resistant cassette (Fig. 13).

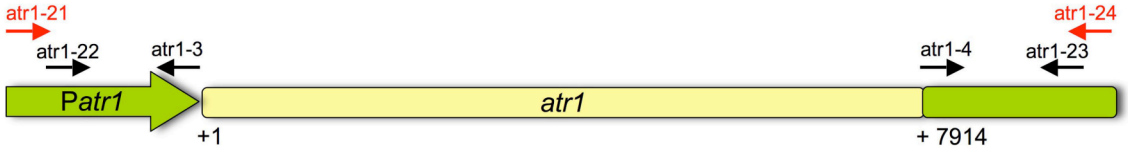


Figure 13: Scheme for the *atr1* gene and the oligos used in the deletion

13.2. *atm1* deletion construct

atm1 deletion was done following the same strategy described above. Two PCR fragments flanking the *atm1* ORF were amplified with atm1-2 and atm1-3, for the 5' homologous fragment, and with atm1-4 and atm1-5 for the 3' one. In this case a hygromycin B resistant gene was used. After *Sfi*I digestion the three fragments were ligated and transformed into *U. maydis*. To check the correct cassette insertion a PCR using atm1-1 and smut3 (1,4 kb fragment) and atm1-6 and smut4 (1,1 kb fragment) was done (Fig. 14).

Figure 14: Scheme for the *atm1* gene and the oligos used in the deletion

13.3. *brh2* deletion construct

For the *brh2* deletion, three cassettes were done, one with the hygromycin B resistant gene, another one with the carboxin one and the other one with the phleomycin one. The 5' homologous fragment was amplified with the brh2-25 and the brh2-28 oligos and the 3' homologous fragment with the brh2-29 and brh2-30. The phleomycin resistant gene was obtained from the pUMA263. After the *Sfi*I digestion and the ligation of the fragments the transformants obtained were checked with the brh2-27 and cbx1 (1,1kb fragment) and brh2-31 and cbx2 (1,6kb fragment) for the carboxin resistant cassette; with brh2-27 and bleo1 (1,3kb fragment) and brh2-31 and bleo2 (1,5kb

fragment) for the phleomycin resistant cassette and with brh2-27 and smut3 (1,1kb fragment) and brh2-31 and smut 4 (1,3kb) for the hygromycin B resistant cassette (Fig. 15).

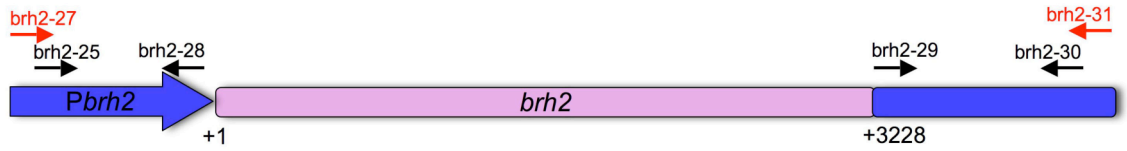


Figure 15: Scheme for the *brh2* gene and the oligos used in the deletion

13.4. Substitution of the *uku70* native promoter by the P_{nar1} inducible promoter

To produce a conditional *uku70*^{nar1} allele, a pair of fragments (one from the promoter region and the other from the *uku70* N-terminal region) were ligated into the pRU2 vector, which had been previously digested with *Nde*I and *Eco*RI. The *uku70* promoter region was amplified with the ku70-2 and ku70-3 oligos flanked with *Pac*I and *Eco*RI sites and the N-terminal region was produced with the ku70-4 and the ku70-5 oligos flanked with *Nde*I and *Pac*I sites, respectively. The resulting plasmid was integrated in the *uku70* locus by homologous recombination after being digested with *Pac*I. To check the correct insertion ku70-1 and cbx2 oligos (1,1kb fragment) and ku70-6 and pnar oligos (1,4kb fragment) were used (Fig. 16).

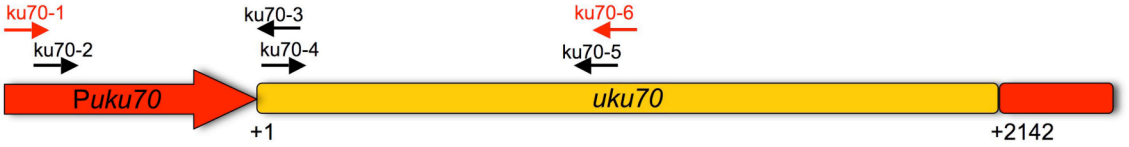


Figure 16: Scheme of the PCR fragments used for the substitution of the *uku70* promoter

13.5. Substitution of the *uku80* native promoter by the P_{nar1} inducible promoter

uku80 promoter substitution by the P_{nar1} inducible promoter was done by cloning two PCR fragments into the pRU2 plasmid (digested with *Nde*I and *Eco*RI). The *uku80* promoter fragment was obtained from the ku80-2 and the ku80-3 oligos and the N-terminal fragment from the ku80-4 and the ku80-5. The first fragment was flanked by *Pac*I and *Eco*RI sites and the second one by *Nde*I and *Pac*I sites. To check the correct insertion of the construct by homologous recombination at the *uku80* locus, ku80-1 and

cbx2 oligos (1,1kb fragment) and ku80-6 and pnar oligos (1,4kb fragment) were used (Fig. 17).

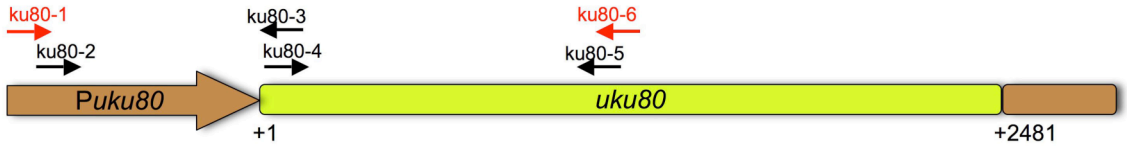


Figure 17: Scheme of the PCR fragments used for the substitution of the *uku80* promoter

14. *Coprinopsis cinerea* strains constructs

C. cinerea strains were obtained from the oidia transformation with the constructs detailed below. The integration of the silencing cassette was checked by the transformants ability to grow in Minimal media. The oligonucleotides used in the constructs appear in each figure and are detailed in table 5.

14.1. pGH-pab construct

To perform the silencing constructs of both genes, first a plasmid with the *gpdII* promoter, a transcriptional terminator and a selectable marker (*pab1* gene) was done. pBS(+)KS plasmid was cut with *Sac*I and *Not*I to be used as a bacterial plasmid pattern. The terminator was obtained from the hygromycin resistant gene from the pNEB-Hyg cut with *Not*I and *Sac*I. After ligation of these two fragments the resulting plasmid was digested with *Sca*I and *Eco*RI and the fragment that contained the terminator was ligated to a *Sca*I/*Eco*RI fragment from the pYSK7 containing the *pab1* gene. pYSK7 was also cut with *Eco*RI and *Bam*HI to isolate the P_{gpdII}, and this was ligated to the plasmid containing the *pab1* gene and the terminator, which had been previously digested with *Eco*RI and *Bam*HI. The resulting plasmid is the pGH-pab and a map of it can be observed in figure 18.

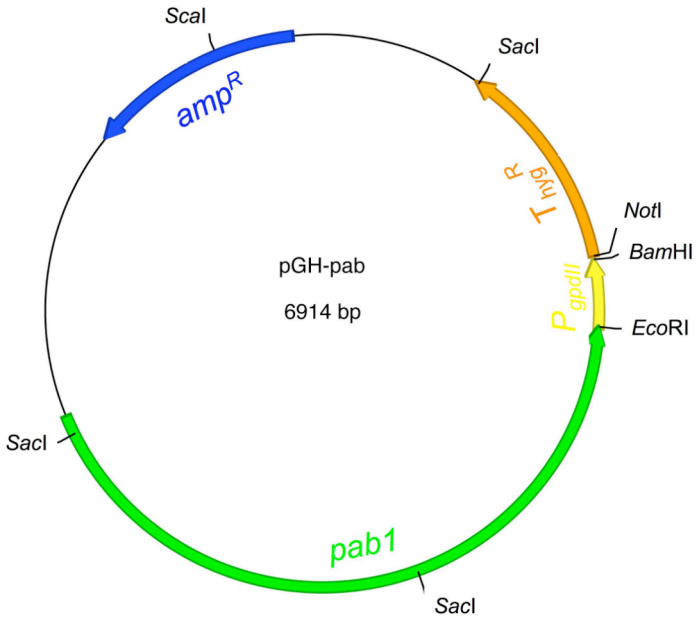


Figure 18: The pGH-pab plasmid

14.2. *atr1* silencing construct

atr1 silencing was done with an antisense construct. For this, two different plasmids containing an antisense fragment of different length were done. In both of them the chosen exon was the largest from *C. cinerea* sequence. The exon was amplified from genomic DNA with atr1c-1 and atr1c-2 oligos (Fig. 19), and was cloned into pGH-pab plasmid as a *Bam*HI/*Not*I fragment. The resulting plasmid, pGH-ATRas-pab was transformed into *C. cinerea* as it has been described.

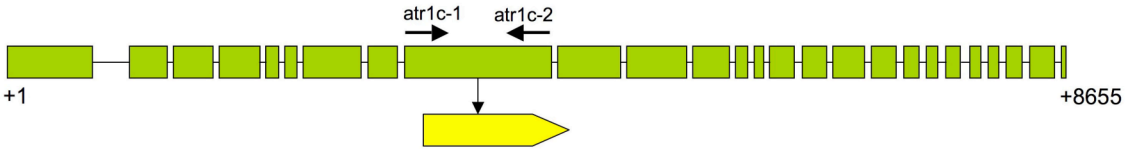


Figure 19: Exon amplified from *atr1* for the antisense construct

For the pGH-ATRasX-pab vector, which had a shorter version of the antisense sequence, pGH-ATRas-pab was cut with *Not*I and *Xho*I. Subsequently it was treated with Klenow to refill the sticky ends so that they could be ligated again (Fig. 20).

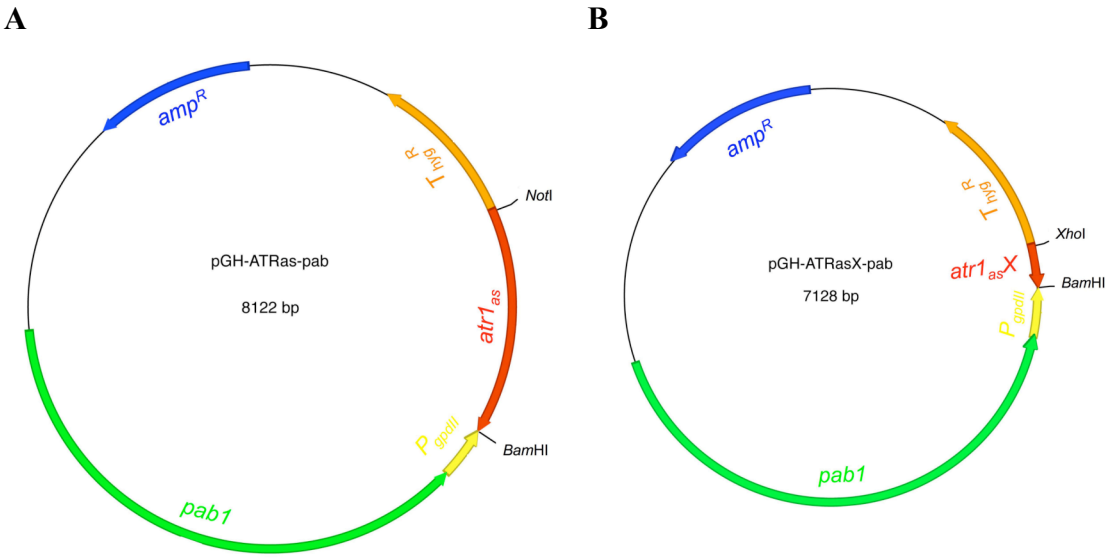


Figure 20: Antisense plasmids created for *atr1* silencing. In A the pGH-ATRas-pab plasmid and in B the shorter pGH-ATRasX-pab.

14.3. *chk1* silencing construct

In the *chk1* silencing, a plasmid carrying an antisense sequence was done. The longest exon was amplified from genomic DNA with chk1c-1 and chk1c-2 and later it was cloned as a *Not*I/*Bam*HI fragment into pGH-pab. The amplified exon as well as the resulting plasmid construct appears in Figure 21.

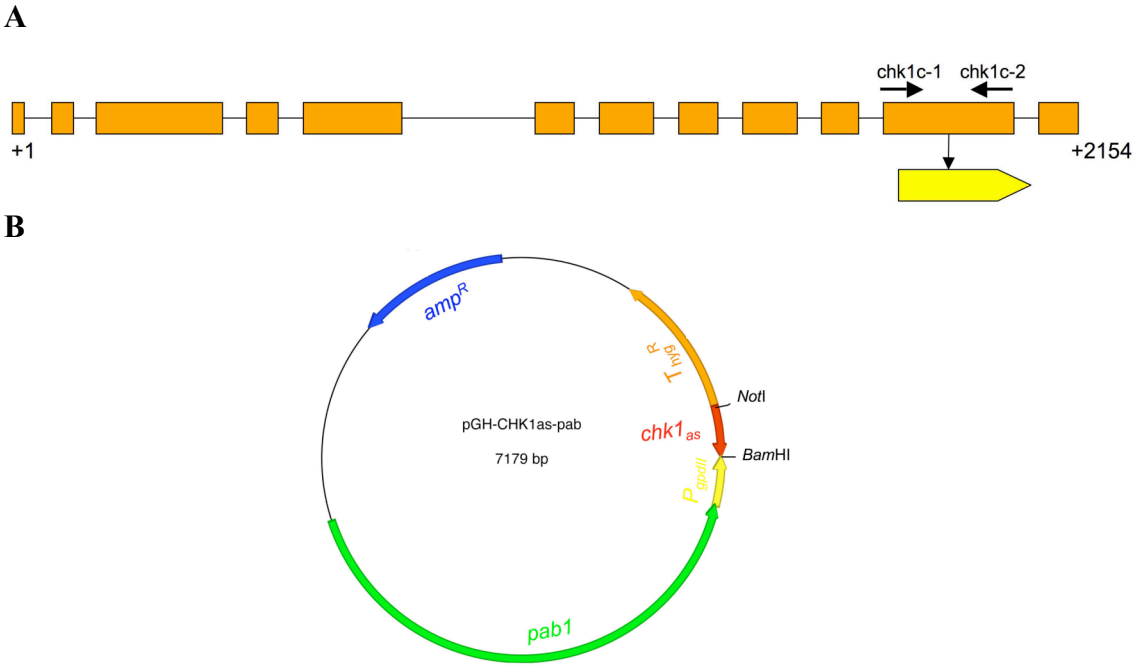


Figure 21: A shows the amplified region from *chk1* and B the pGH-CHK1as-pab plasmid.

Table 5: Oligos used in this study. In bold, the restriction enzyme sites inserted in the sequence are denoted.

Oligo name	Sequence 5'-3'	Use
bleo1	gtgtccggtgcatttcgccttctcggcg	Check colonies
bleo2	aaacctcgaaatcattcctactaagat	Check colonies
cbx1	tctgggtttcgcgagagatctcacagagca	Check colonies
cbx2	aattgcacagatcaagaaggacatggccgt	Check colonies
pnar1	ggtgaatagtgagaacagtctcgatcactctg	Check colonies
smut3	attcacgttttgtagcacacgactcacatc	Check colonies
smut4	caccacccaatcgacgcggaaggcaaccca	Check colonies
atr1-3	ggt**ggccatctaggcc**ttccttaggcttggacactggagatcagt	Constructs
atr1-4	ata**ggcctgagtggcc**acggtttgcagctgcatacagtaggatat	Constructs
atr1-21	gccggaaggcagatggacaacacaatcaca	Check colonies
atr1-22	**ttaattaa**gcagatccactgctgaacgggtttc	Constructs
atr1-23	**ttaattaa**ggaactcatcagcgtgtggaaccga	Constructs
atr1-24	aagacgagagcgcctggcaggtagtgatcg	Check colonies
atm1-1	tacgattgagccaatcttggtggaagatcc	Check colonies
atm1-2	**ttaattaa**ttctttgcaaagcttttcgtggttt	Constructs
atm1-3	ggt**ggccatctaggcc**gtggctttctgatggcgtcgacctatgtt	Constructs
atm1-4	ata**ggcctgagtggcc**tcggctcgagatggaaggaattcacgatt	Constructs
atm1-5	**ttaattaa**tacaaatacaacgtcgaaatctcgg	Constructs
atm1-6	acacgttatcttgcgcgcaaattccaacag	Check colonies
brh2-25	**ttaattaa**acagtcgtcagtacgcatgcatgcc	Constructs
brh2-27	aaggtggggtggtcaacgtggctctgcata	Check colonies
brh2-28	cat**ggccatctaggcc**tcgagtggtctgaattgagttgggaaagg	Constructs
brh2-29	cta**ggcctgagtggcc**ccagatcggttctttcttggaacacgtcg	Constructs
brh2-30	**ttaattaa**cccgttccgcgtcgcaacacagcag	Constructs
brh2-31	aggttcaagcgcagtcgagctttgatagtc	Check colonies
ku70-1	aaaggaagagaccgaccggcaaaagtggcc	Check colonies
ku70-2	**ttaattaa**cgggcaaaccttgcagcctcaacct	Constructs
ku70-3	**caattg**aataccgcacaagttggagtatgtggc	Constructs
ku70-4	**catatg**cccaaggcttactttgtcaacaagcgc	Constructs
ku70-5	**ttaattaa**cacaacacgtttgggtgtctcgcgc	Constructs
ku70-6	gcagtcgtcgtcgacgattaccttgacagg	Check colonies

ku80-1	gcgagccgaagtctgtgtgatgaggtcgaa	Check colonies
ku80-2	**ttaattaa**agcgacacagaggccaataatggtc	Constructs
ku80-3	**gaattc**atgcggatgtggcggccatgacaaggg	Constructs
ku80-4	**catatg**agtgtcgaatccaacacgctcacgctc	Constructs
ku80-5	**ttaattaa**cgggggctacaggtgtcgaggtggg	Constructs
ku80-6	tttgcatccgactgaggtggaagcggctca	Check colonies
atr1c-1	gcgatattccagacctcattgtctgtcgca	Constructs
atr1c-2	tac**ggatcc**actgagaatcttcttgtcatgcgt	Constructs
chk1c-1	tcgcaaacgcaatcgggccctcgatatgtgccg	Constructs
chk1c-2	atg**ggatcc**tcatctctcgccatgccgcagaag	Constructs
Telomeric	ttagggttagggttagggttagggttaggg	2D gel
subtelomeric-1	aagcgagattcggtcgcagcgttggcagcacac	Southern probe
subtelomeric-2	ttgctggtgtccccacccagagcatatactcgt	Southern probe
tub RT-1	cgagatgaccttctcgtcgt	RT-PCR
tub RT-2	aacatcaccacggtacagca	RT-PCR
ku70 RT-1	tgcatacagaagacgccaag	RT-PCR
ku70 RT-2	gtgatggattggggtagtgg	RT-PCR
ku80 RT-1	aaatgtgtcgccaaaggttc	RT-PCR
ku80 RT-2	tgttccgatcgttttcatca	RT-PCR
benA RT-1	cttgcttcgagcctggtaac	RT-PCR
benA RT-2	tcaccacggtagaggagagc	RT-PCR
atr1-5	cagctgaactggaagcacag	RT-PCR
atr1-6	gggaacatcgggagaatctt	RT-PCR
chk1-3	attctgccgctatctggatg	RT-PCR
chk1-4	ggttcacgttcagcataccc	RT-PCR

Results

Atr1 and the DNA damage response pathway in *Ustilago maydis*

1. Characterization of Atm1 and Atr1 in *Ustilago maydis*

1.1. Atr1 and Atm1 identification in *U. maydis*

To identify Atr1 and Atm1 homologues a homology search was done through the whole *U. maydis* genome by using the BLAST program. SpRad3 and ScMec1 sequences, ATR homologue proteins from *S. pombe* and *S. cerevisiae* respectively, and SpTel1 and ScTel1, ATM homologue proteins in the aforementioned organisms, were used as queries. The result of these analysis were two proteins, um01110 and um15011.2, which had previously been identified as entries in the manually annotated Munich Information Center for Protein Sequences *U. maydis* database (http://mips.gsf.de/genre/proj/ustilago). A phylogenetic analysis made possible to include um01110 within the ATR protein family and um15011.2 within the ATM one (Fig. 22).

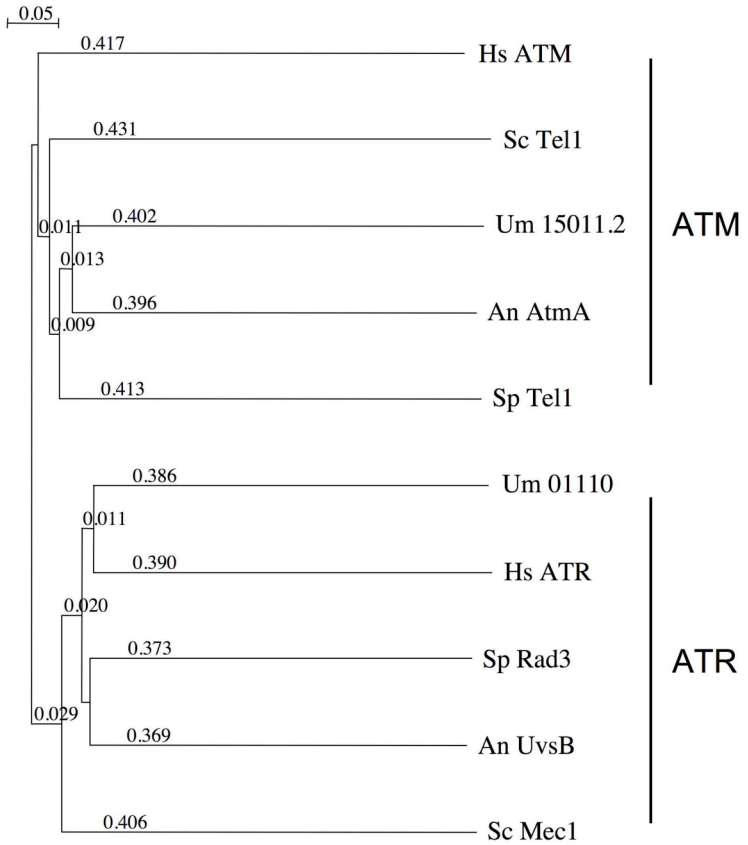
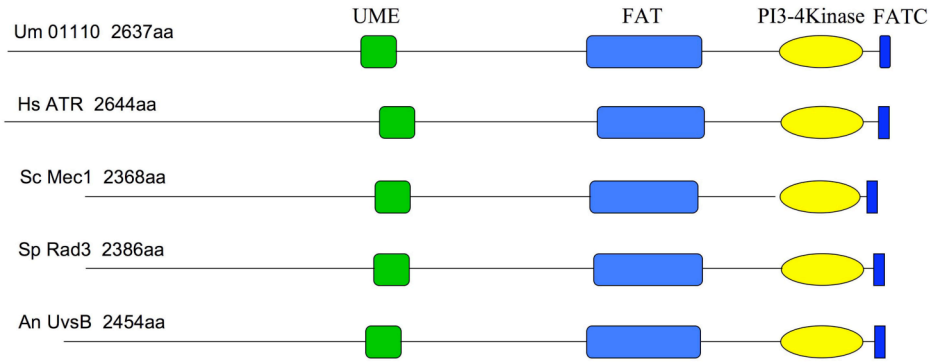


Figure 22: Dendogram obtained from the alignment of ATR-like and ATM-like proteins done by ClustalW2 (**http://www.ebi.ac.uk/Tools/msa/clustalw2/**). Sequences used I the analysis came from *Homo sapiens*, *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Schizosaccharomyces pombe*.

Um01110 is a protein of around 2600 amino acids and a molecular weight of 292kDa, where all the characteristic domains of the family are present. Um01110 belongs to the PhosphatidylInositol 3-Kinase related Kinases (PIKKs) family (Tibbetts and Abraham, 2001) and its kinase domain is located in the C-terminal region. This domain phosphorylates serine or threonine residues from other proteins (Hartley et al., 1995; Hunter, 1995). Apart from this, there are two more characteristic domains that are a hallmark of the PIKKs, the FAT and FATC domains (FRAP/ATM/TRAPP conserved domains). These domains can only be found in this protein family and always appear together, therefore a possible role for the proper function of the PI3-kinase domain due to a probable interaction between them has been suggested (Bosotti et al., 2000). Apart from these three domains, ATR-like proteins present another domain named UME, which is found in nucleolar proteins (UVSB PI3-kinase/MEI-4/ESR1 conserved domain) (Staub et al., 2004) (Fig. 23A).

um15011.2 is a 2-exons ORF that codes for a protein of around 3030 amino acids and a molecular weight of almost 334kDa. As ATR-like proteins, ATM belongs to the PIKKs family. This family, as described before, is characterized by the presence of three different domains: the kinase domain at the C-terminal and surrounding it the FAT and FATC domains. ATM-like proteins share another domain called TAN (Tel1/ATM N-terminal motif), which has been described to be essential for telomere length maintenance and DNA damage response (Seidel et al., 2008). All these domains could be found in Um15011.2 (Fig. 23B).

A



B

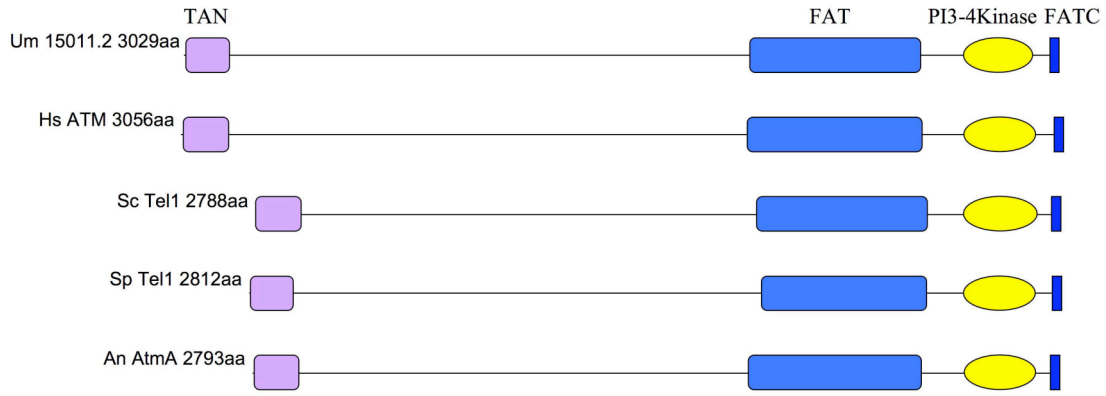


Figure 23: *U. maydis* Atr1 and Atm1. A) Schematic representation showing the domain architecture of ATR-like proteins in different organisms. B) Scheme showing the different described domains from the ATM-like proteins.

Due to the sequence similarity and the presence of all the conserved family domains, form now on, Um01110 will be designated as Atr1 and Um15011.2 as Atm1.

1.2. Atr1 deletion phenotype in *U. maydis*

ATR family members in other organisms have shown to have a role in DNA damage response (Shiloh, 2001; Zhou and Elledge, 2000). They are considered as part of the sensing proteins from the DNA damage checkpoint pathway (Sancar et al., 2004). ATR triggers the cellular response against single stranded DNA breaks and DNA replication stress (Durocher and Jackson, 2001). In other organisms deletion of ATR-like proteins resulted on an increased sensitivity of the cells not only to UV radiation or agents that inhibited the DNA replication but also to other genotoxic agents as bleomycin, MMS and γ-radiation (Bentley et al., 1996; Pike and Heierhorst, 2007; Seaton et al., 1992). Atr1 activity in *U. maydis* was analyzed by using the construct described in figure 13 to transform haploid cells. Cells lacking *atr1* were viable, indicating that *atr1* is not essential although they presented a slow growth rate and a high sensitivity to genotoxic agents. *atr1Δ* sensitivity to DNA damage agents was compared to other strains deleted in genes involved in DNA repair as *chk1*, homologue gene of the well-known DNA damage checkpoint kinase Chk1 (Perez-Martin, 2009), and *brh2*, *U. maydis* BRCA2 homologue involved in DNA repair by homologous recombination (Kojic et al., 2002). As it can be observed in figure 24, *atr1Δ* cells were the most sensitive of all the strains against the DNA insults tested. This showed that the described role for *atr1* in DNA damage response was also conserved in *U. maydis*.

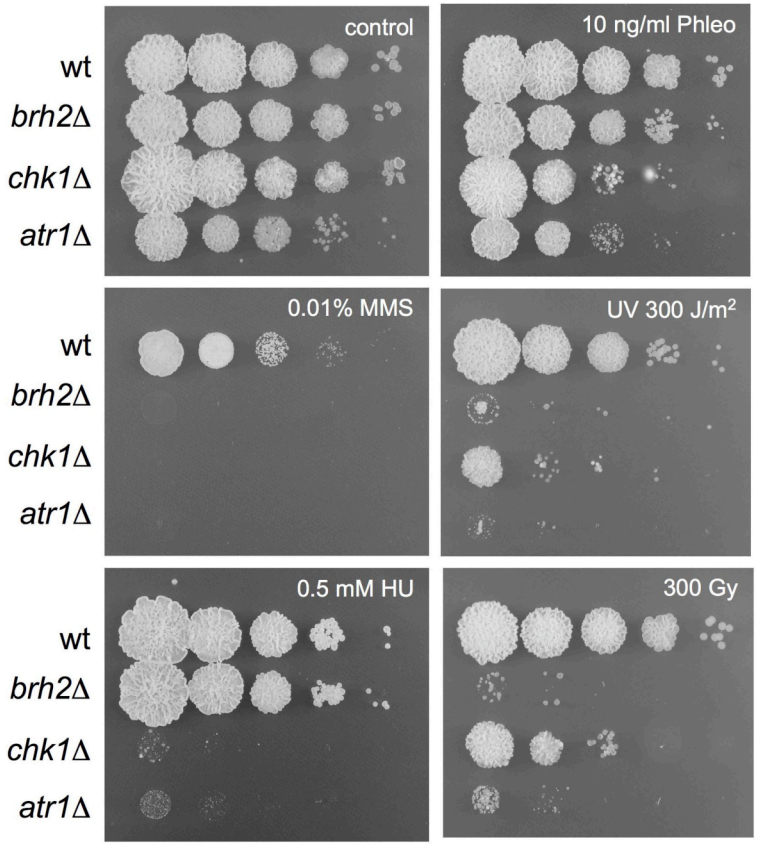


Figure 24: Phenotype of cells lacking *atr1* compared to wild-type, *chk1Δ* and *brh2Δ*. Strains used in this assay were UCS9, UCM350, UMP122 and UCM565 and the picture was taken after a 2-days incubation.

1.3. Atm1 essentiality in *U. maydis*

As ATR-like proteins, ATM-like proteins are involved in DNA damage checkpoint. ATR and ATM are considered the sensing proteins from the DNA damage response pathway that activate Chk1. ATM can bind directly to the DNA and is mainly involved in DSBs recognition (Rouse and Jackson, 2002). In *S. cerevisiae* and mouse it has been described that at least one of these proteins is essential and in both cases it happened to be ATR (de Klein et al., 2000; Kato and Ogawa, 1994). This essentiality and the partial redundant roles described for both proteins leaded to consider ATR as the housekeeping member of this duo (Abraham, 2001). When characterizing *atm1* in *U. maydis* a deletion cassette similar to the one used for *atr1* was created (Fig. 14) and haploid wild type cells were transformed. No transformants were obtained suggesting that *atm1* would be essential. Therefore a diploid strain was transformed. From this transformation, two independent clones $atm^{+/-}$ were chosen and were subsequently used to infect maize plants in order to obtain diploid teliospores. From the tumours produced

in the plant, teliospores could be isolated and these were subsequently germinated. In *U. maydis*, germination is coupled to meiosis. After germination and subsequent meiosis, no haploid cells carrying the *atm1Δ* cassette could be recovered. This suggested that *atm1* was essential in *U. maydis*.

2. Atr1 is required for the activation of Chk1 in response to DNA damage in *U. maydis*

In other model systems, ATR together with the other PIKK, ATM, phosphorylate Chk1 and Chk2, considered as the "effector kinases" in the DNA damage response pathway (Melo and Toczyski, 2002; Nyberg et al., 2002). These kinases are primordial for the connections between the DNA-damage checkpoint pathway and the cell cycle machinery (Nyberg et al., 2002). In *U. maydis* only a Chk1 ortholog is present, which is required for sensing DNA double strand breaks and behaves as Chk1 homologues in other organisms (Perez-Martin, 2009). To check if Atr1 regulation over Chk1 was also conserved in *U. maydis* and define *atr1* and *chk1* relationship, firstly a *chk1Δ* and *atr1Δ* double mutant was constructed and its sensitivity towards genotoxic agents as UV radiation was tested (Fig. 25). As control for this epistasis analysis an *atr1Δ brh2Δ* double mutant strain was created. As mentioned previously, *brh2* is the BRCA2 homologue in *U. maydis*, and until now, no data about a common pathway between these two proteins has been described.

The results suggested that *atr1* and *chk1* worked in the same pathway since double deletion mutants presented the same phenotype as the single *atr1Δ* mutant, while *brh2* seemed to act in a different pathway to *atr1* as their effects were additive.

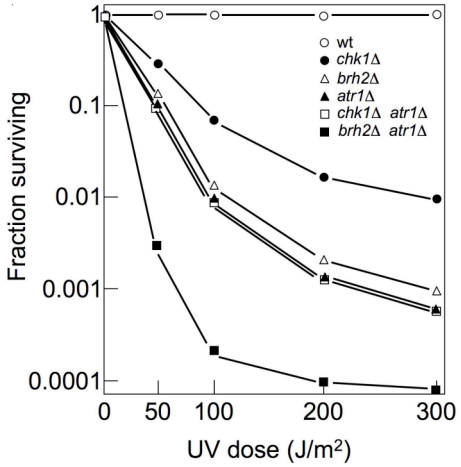


Figure 25: Epistasis analysis between *atr1* and *chk1*.

Secondly, Atr1 requirement for Chk1 phosphorylation in response to DNA damage, which has been broadly described in other systems, was studied (Guo et al., 2000; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). Two main DNA-damage signals can cause Chk1 phosphorylation in *U. maydis*: the presence of double-strand breaks that could be induced by phleomycin treatment, and the presence of single-strand DNA tracts, hallmark of replication stress which we could mimic by HU treatment. To analyze the phosphorylation status of Chk1, a mobility shift assay of Chk1 in *U. maydis* was used (Perez-Martin, 2009). For this, wild-type and *atr1Δ* cultures of strains carrying a *chk1-T7* allele were treated with phleomycin or HU and Chk1 phosphorylation was analyzed by Western blot (Fig. 26). It was observed that in absence of Atr1 the mobility shift of Chk1 was abolished in cells treated with HU, suggesting that Chk1 phosphorylation in response to this stimulus was fully dependent on the presence of Atr1. In contrast, in cells treated with phleomycin, both Chk1 states could be observed, showing probably that both DNA damage response pathways triggered the response to this genotoxic agent.

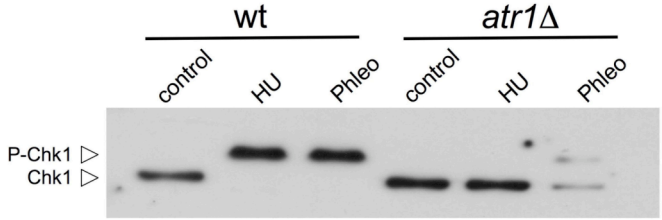


Figure 26: In vivo phosphorylation of Chk1 in response to DNA damage depends on Atr1.The strains used were UMP162 and UMP207.

It has been proven that Chk1 phosphorylation seems to be essential for its activation, and this is coupled with its location change from the cytoplasm to the nucleus in response to DNA damage (Dunaway et al., 2004; Jiang et al., 2003). Therefore, subcellular localization of Chk1 was studied in *atr1Δ* strains treated with genotoxic agents. In this experiment wild-type and *atr1Δ* strains carrying a green fluorescent protein (GFP)-tagged Chk1 were observed in presence of either HU or phleomycin (Fig. 27).

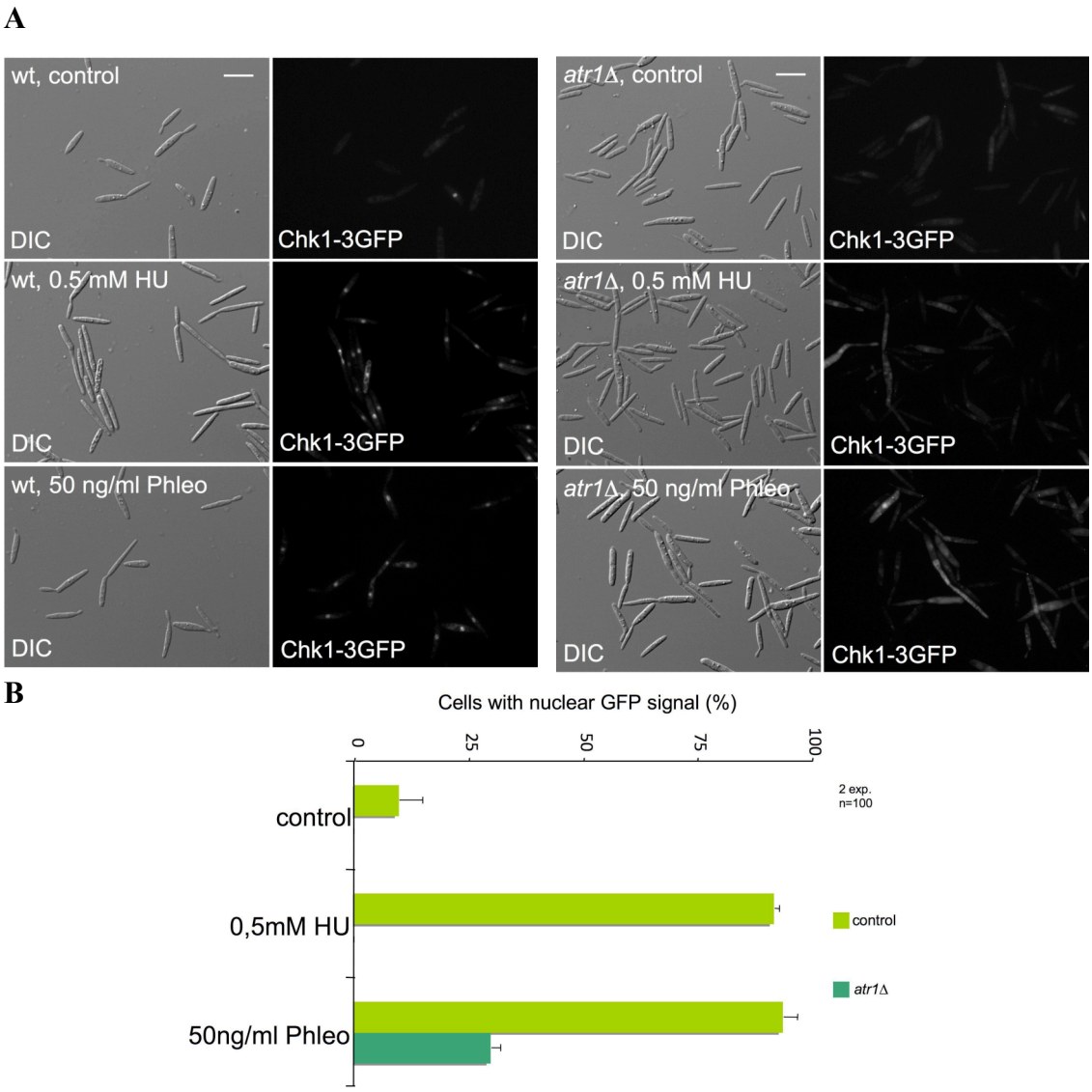


Figure 27: Chk1 subcellular location change in response to genotoxic agents depends on Atr1.Strains used here were UMP111 and UCS15.

In wild-type cells a clear nuclear localization of Chk1 could be seen in presence of these DNA damage agents, contrarily to what was observed in the absence of Atr1, when Chk1 failed to localized in the nucleus in presence of DNA damage. This absence in the nuclear localization of Chk1 was clearer in cells treated with HU than in those treated with phleomycin, agreeing with what had been observed in the Chk1 phosphorylation western blot. So it seemed that the ATR-Chk1 axis described in other model organisms to have a role in DNA damage response was also conserved in *U. maydis*.

In response to DNA damage, Chk1 gets active and arrests the cell cycle in G_2 phase. This G_2 cell cycle arrest can be easily distinguish in *U. maydis* by the presence of long buds (bud growth is produced at G_2 phase in *U. maydis*) (Perez-Martin et al.,

2006). If this arrest is sustained the strong polar growth associated to G_2 phase produces long filaments. We observed that *atr1Δ* cells accumulated elongated cells during growth in rich media, which resembled the filaments obtained after a sustained G_2 cell cycle arrest. The population of these elongated cells increased with the culture time, arising a 30% of the total population after 24 hours of growth in absence of any genotoxic treatment. We hypothesized that this phenotype was the consequence of the inability of these cells to cope with the basal DNA damage, which would accumulate until a stronger signal, not transmitted through the Atr1 cascade was produced. We observed that in the *atr1Δ chk1Δ* cells, the number of elongated cells decreased to an 8% (Fig. 28). So it seemed that the elongated phenotype that could be seen in the *atr1Δ* indicated a cell cycle arrest probably due to the accumulation of DNA damage, and this phenotype was partially suppress with the deletion of *chk1*.

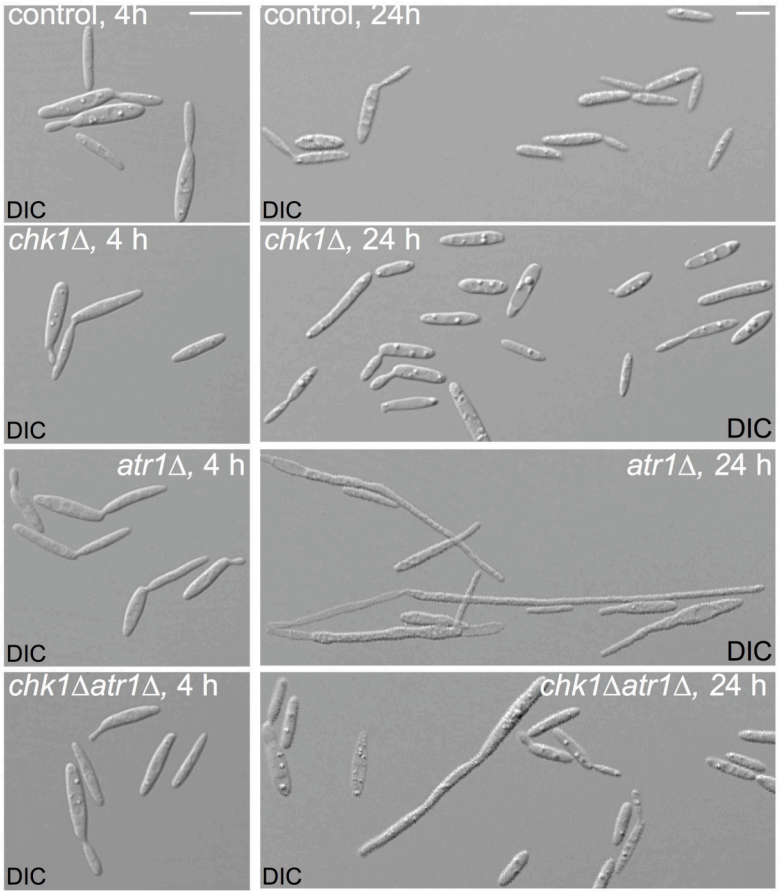


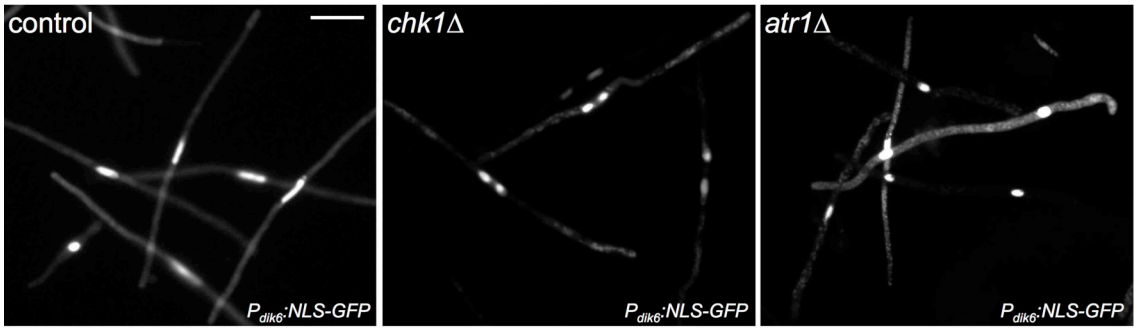
Figure 28: *atr1Δ* induces elongated cell formation related to cell cycle arrest. Strains used in this assay were wild type, UMP122, UCS1 and UCS22. Bar = 10μm.

Atr1 and Chk1 in dikaryon development

1. Atr1-dependent phosphorylation of Chk1 is required for b-dependent cell cycle arrest

The formation of an active bW-bE heterodimer in *U. maydis* results in a sustained cell cycle arrest in G_2 phase, and this arrest is controlled by Chk1 (Mielnichuk et al., 2009). As we have previously seen, Atr1 controls Chk1 activation in response to DNA damage. Therefore we wondered if Atr1 would also be controlling Chk1 activity during b-induced cell cycle arrest. For this, *atr1* gene was deleted in a strain where the expression of the b-complex heterodimer homeoprotein could be induced (AB33). Due to the elongated phenotype of *atr1Δ,* a GFP-nuclear localization signal fusion under the *dik6* promoter was used in order to quantify only the filaments produced after the b-induction. *dik6* promoter is specifically activated by the bW-bE heterodimer (Flor-Parra et al., 2006). AB33 strain carries the b-complex under the inducible promoter P_{nar1}. To carry out this experiment, firstly the cells were grown under non-inducing conditions (YPD media) and afterwards they were changed to inducing conditions ($MMNO_3$ media) for several hours. It was observed in the control strain that almost all the b-expressing cell population had a single nucleus, while in the *atr1Δ* strain around a 50% of the cells presented more than one nucleus (Fig. 29).

A



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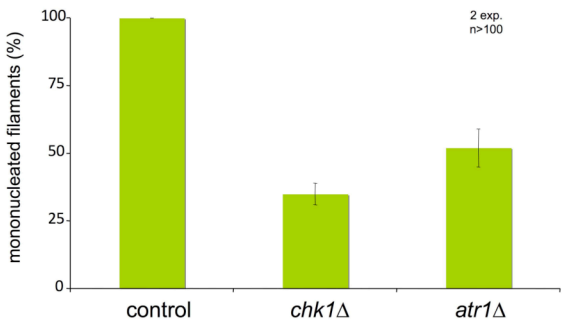


Figure 29: A) Images of AB33 strains carrying an NLS-GFP fusion under the *dik6* promoter in wild-type (UCS20), *chk1Δ* (UMP112) or atr*1Δ* (UCS21) background. Bar = 15µm. B) Quantification of all the mononucleated filaments after 24 hours of b-induction.

This meant that these cells were unable to arrest before entry into mitosis, which suggested that Atr1 could also be involved in Chk1 activation in presence of the b-complex. To assess this hypothesis an analysis of Atr1 role in the Chk1 phophorylation state when the b-proteins were present was done. For this an AB33 strain carrying an endogenous *chk1-T7* allele was deleted in *atr1* and compared to a control AB33 *chk1-T7* strain. These strains were grown under inducing conditions ($MMNO_3$) for 4 hours and samples were taken every 2 hours. Protein extracts were inmunoprecipitated with a commercial anti-T7 antibody, then were run on a SDS-PAGE gel and finally were inmunoblotted with an anti-T7 antibody. Consistently with our hypothesis, no Chk1 phosphorylation was observed in cells lacking *atr1* and expressing the b-proteins (Fig. 30).

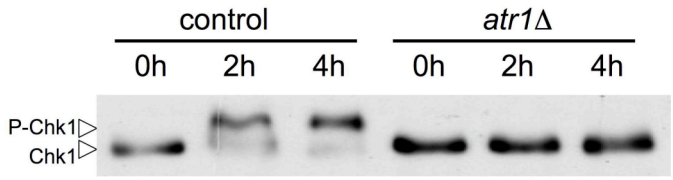


Figure 30: Chk1 phosphorylation during b-dependent filamentation depends on Atr1. AB33 strains with an endogenous *chk1-T7* fusion on control (UCS31) or atr*1Δ* (UCS32) backgrounds.

Given that phosphorylation seems to be required for Chk1 activation and nuclear accumulation, Atr1 role in Chk1 dependent localization upon induction of the b-proteins was also investigated. Here, AB33 strains carrying a Chk1-GFP fusion were grown on filament-inducing conditions for 6 hours. As it had been previously described, Chk1 activation in the filament is a transient response and only occurs shortly after the b-induction, which means that it can only be seen in the nucleus in small filaments (Mielnichuk et al., 2009). This observation was confirmed again and only in the control cells that presented short filaments, a clear Chk1 nuclear localization could be observed, while in atr*1Δ* cells, this nuclear accumulation of GFP signal could not be observed at any stage of the filament development (Fig. 31).

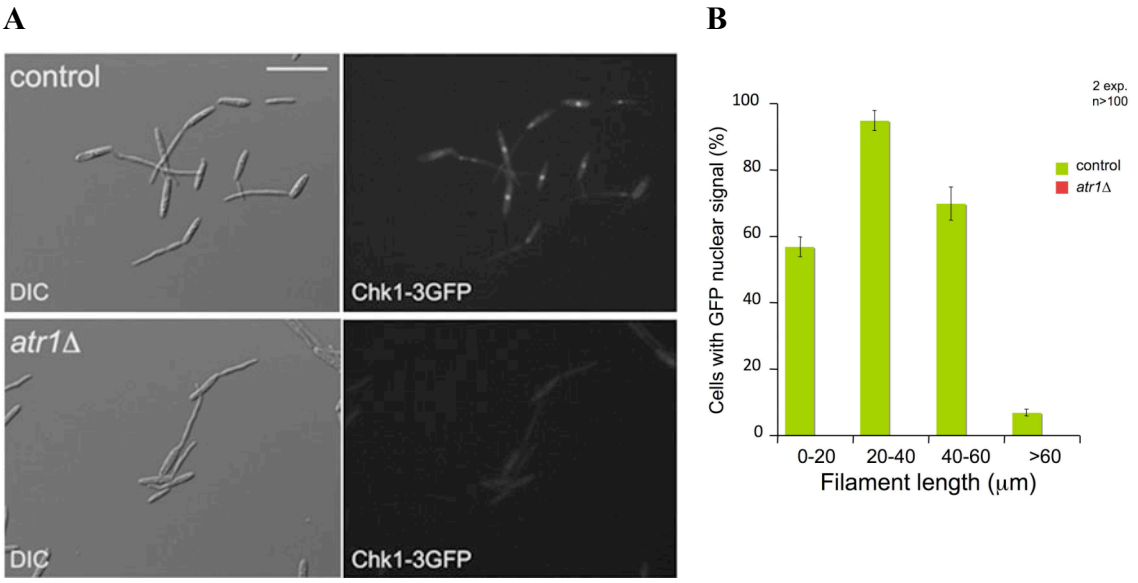


Figure 31: Chk1 nuclear localization on b-induced cells depends on *atr1*. AB33 cells carrying a Chk1-3GFP protein fusion on control (UMP133) or *atr1Δ* (UMP208) backgrounds. Bar = 30μm

Taking all these results together, it seemed that the Atr1-Chk1 pathway had an essential role for the correct establishment of the infective filament in *U. maydis*.

2. DNA damage and b-dependent Chk1 activation rely on the same residues

It had been described that ATM and ATR proteins, phosphorylate serine or threonine residues followed by glutamine (Kim et al., 1999). A previous work in *U. maydis* Chk1 characterization in response to DNA damage had shown that Chk1 activation involved phosphorylation at two residues located in the regulatory domain (Thr-349 and Ser-448) and that mutant isoforms containing Ala in place of these residues could not be activated in response to DNA damage signals (Perez-Martin, 2009) (Fig. 32A). To determine whether these phosphorylation sites were also essential for the b-dependent cell cycle arrest, a phosphorylation refractory Chk1 allele was inserted on an AB33 strain. For this, AB33 cells carrying a *chk1*$^{T394A\ S448A}$*-T7* fusion integrated at its native locus were grown on *b*-inducing conditions and samples were taken every two hours. As it is shown in figure 32B, upon b-induction, no Chk1$^{T394A\ S448A}$ phosphorylation could be seen, which showed that these sites were also essential for the b-dependent Chk1 activation.

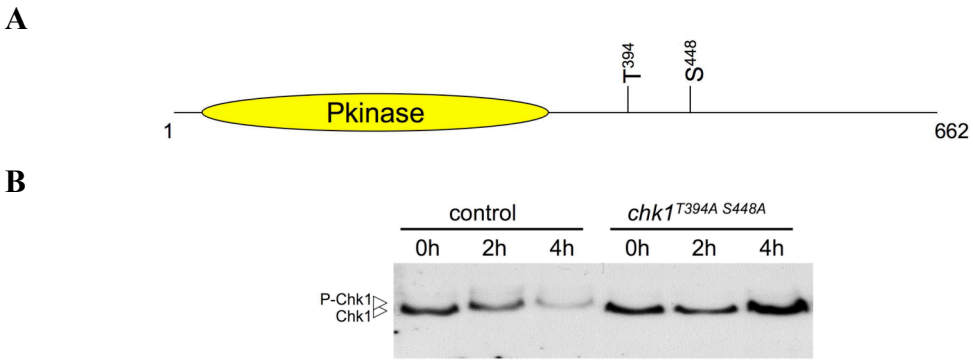


Figure 32: Threonine 394 and Serine 448 are essential in Chk1 activation after b-proteins induction. A) Chk1 scheme showing the location of T394 and S448. B) Western blot where the absence of Chk1 phophorylated form in the *chk1*$^{T394A\ S448A}$ strain can be observed.

To confirm that these residues were essential in the b-dependent Chk1 activation that leads to a cell cycle arrest, we decided to analyze the effect of this non-phosphorylatable Chk1 allele on the cell cycle arrest under b-inducing conditions. As it can be observed in figure 33A almost 60% of the cell population could avoid this arrest. Finally to see if these mutations could abolish completely *chk1* activity, compatible strains carrying a *chk1*$^{T394A\ S448A}$ allele were studied for their ability to infect maize plants (Fig. 33B). The result of this infection was very similar to what had previously been observed on a *chk1Δ* infection.

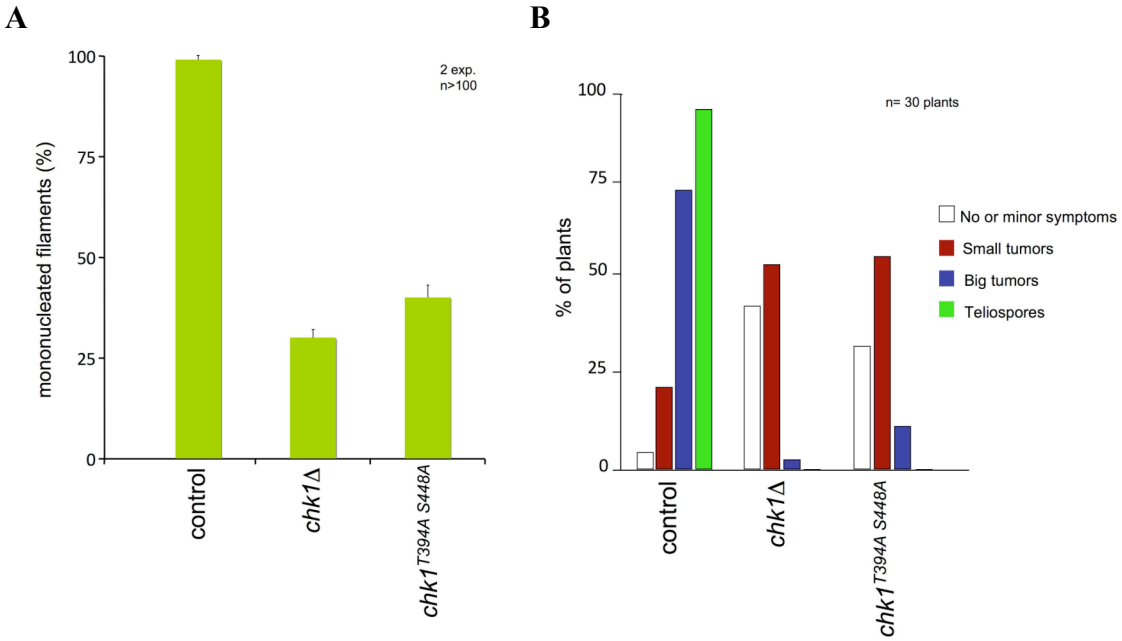


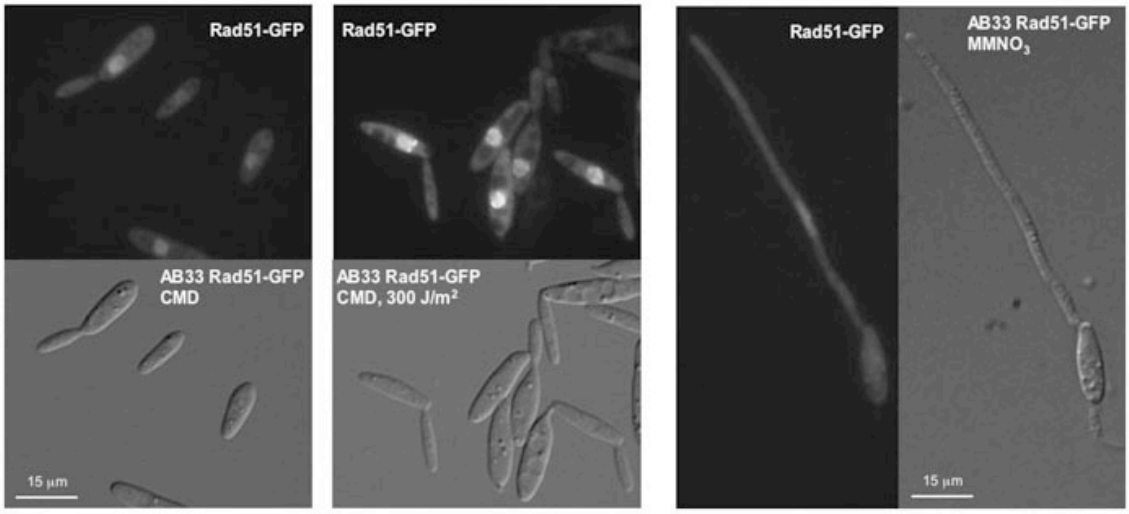
Figure 33: A nonphosphorylatable Chk1 form mimics a *chk1Δ* with respect to b-dependent cell cycle arrest and *in planta* proliferation. A) Strains used were UCS20, UMP112 and UMP183. B) Strains used in the crosses were: FB1xFB2, UMP122xUMP129 and UMP190xUMP191.

All these results suggested that phosphorylation at T394 and S448 residues is also essential for b-dependent Chk1 activation and that a change for non-phosphorylatable residues results in a *chk1Δ* phenocopy.

3. b-dependent Chk1 activation does not correlate with a DNA damage response

Atr1-Chk1 axis activation has broadly been described to occur in response to DNA damage. The activation of this axis leads to a G_2 cell cycle arrest in order to give enough time to the cell to solve the damage before entering into mitosis (Liu et al., 2000). We have shown in "Atr1 and the DNA damage response pathway in *Ustilago maydis*" that Atr1 is an upstream regulator of Chk1 activation in response to DNA damage in *U. maydis*. And previously it had been demonstrated that in *U. maydis* Chk1 activation in response to DNA damage leaded to a G_2 cell cycle arrest (Perez-Martin, 2009). Therefore we wondered if this b-induced G_2 cell cycle arrest was related to the presence of damage on the DNA. To assess this, Rad51 was used as reporter. Rad51 is involved in DNA repair by homologous recombination. Upon treatment with DNA damage agents, Rad51 binds to the damaged DNA and this can be followed by the formation of foci in the nucleus (Kojic et al., 2005). A GFP-Rad51 fusion was inserted on an AB33 strain and Rad51 foci were studied after b-induction. As control, the same strain grown in non-inducing conditions (CMD) was treated with UV light. As it can be seen in figure 34, Rad51 foci were observed in almost an 80% of the cell population treated with UV radiation, while no foci could be notice upon b-homeodomain proteins induction.

A



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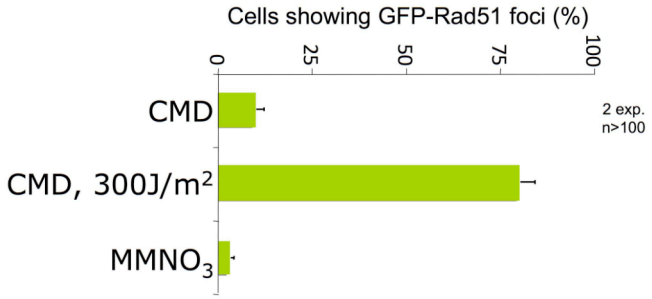


Figure 34: b-dependent cell cycle arrest is not dependent on massive DNA damage. A) Rad51 localization in control conditions, UV treated cells and b-inducing media. B) Quantification of cells with Rad51 foci in their nucleus. UMP168 was the strain used.

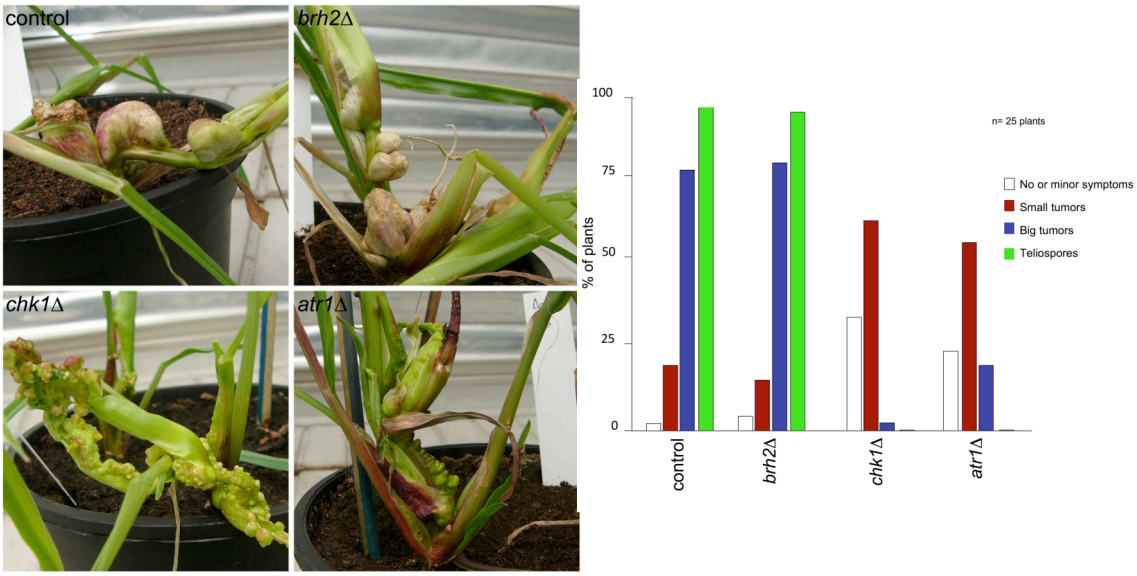
This result meant that Atr1-Chk1 axis activation after the b-induction was not due to the presence of DNA damage, or at least not to the type of damage that would be recognized by Rad51. Further argumentation on this observation will be done in the discussion.

4. Atr1 and Chk1 are required for full virulence in *U. maydis*

As we have seen, in *atr1Δ* strain the cell cycle arrest during dikaryon establishment was impaired. As defects in dikaryon establishment had previously been attributed to be the cause of a defective plant infection (Mielnichuk et al., 2009), we analyzed the ability of *atr1Δ* to infect corn plants. Therefore, maize plants were infected with wild type, *chk1Δ* and *atr1Δ* strains to check their pathogenicity during the infection process. In the infection analysis a *brh2Δ* strain was also used as control of a

strain impaired in the ability to cope with DNA damage (Fig. 24). *U. maydis* infection results in anthocyanin pigment production by the plant and the formation of tumors that are filled with proliferating fungal cells that eventually differentiate into black teliospores (Banuett and Herskowitz, 1996). In the infection it was found that >25% of the plants infected with the *chk1Δ* or the *atr1Δ* strains showed no or minor symptoms such as chlorosis and anthocyanin pigment production. As *atr1Δ* and *chk1Δ* are impaired in the infective filament establishment, this reduced symptomatology could be related to a penetration defect. In addition, only a few of the infected maize plants showed big tumors. No matter the size of the tumor, in all the cases no teliospore formation could be observed (Fig. 35A). This defect in teliospore formation could be due to a failure in the fungal development inside the plant. To see the development of the fungi inside the plant a Chlorazole Black E staining of the infected plants was performed (Fig. 35B). Two weeks post-infection a broad development of the fungi inside the plant as well as the presence of spores could be observed on plants infected with wild-type compatible strains. In the *atr1Δ*-infected plants, no spores were seen and the hyphae inside the plant were very few, possibly indicating, as it had previously been proposed for *chk1Δ*, a poor colonization of the fungi. One possible explanation for this little infection observed in the strains impaired in the DNA damage response could be that their development inside the plant might be affected by the stress induced by the plant defense system. Although this explanation was quickly rejected as *brh2Δ* strain behaved normally (Fig. 35A).

A



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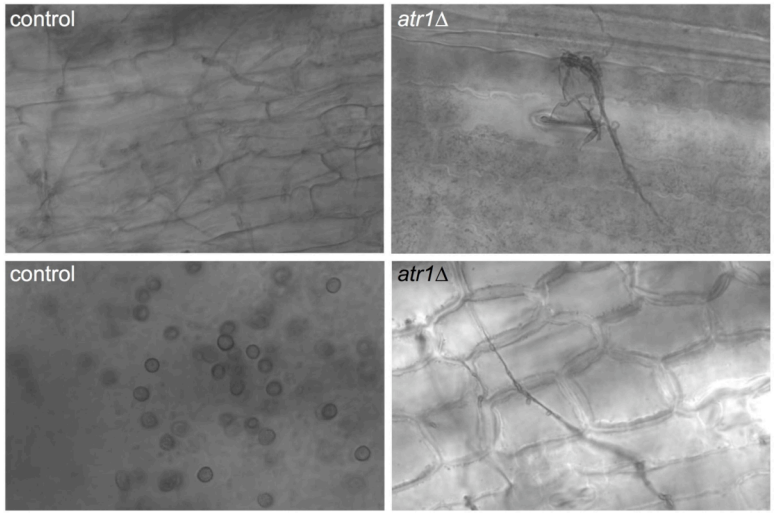


Figure 35: A) Tumors observed 16 days after infection on maize plants infected with wild-type (UCM350 x UCM520), *brh2Δ* (UCM565 x UCM575), *chk1Δ* (UMP122 x UMP129) or *atr1Δ* (UCS9 x UCS10) compatible strains and the quantification of the produced effects. B) Chlorazole Black E staining of 16 days post-infection maize plants, where in wild-type strains spores can be observed.

Interestingly, the tumors produced by the *chk1Δ* and the *atr1Δ* strains showed small shoot-like structures (Fig. 36). These results suggested that Chk1 and Atr1 beyond their role in signaling of DNA damage might have an essential role for the pathogenesis of *U. maydis*.

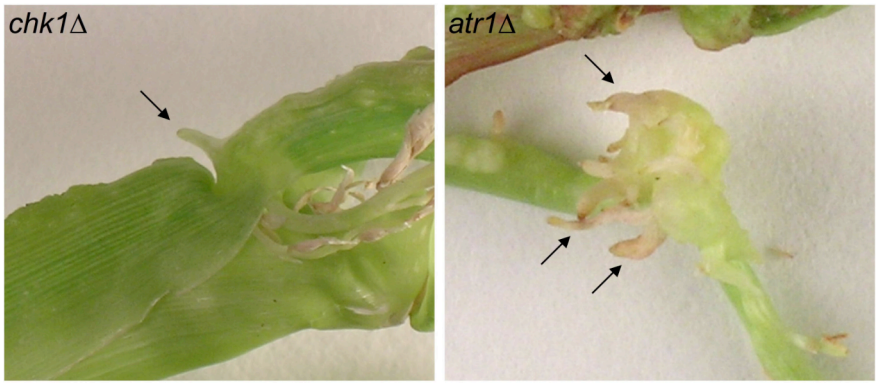


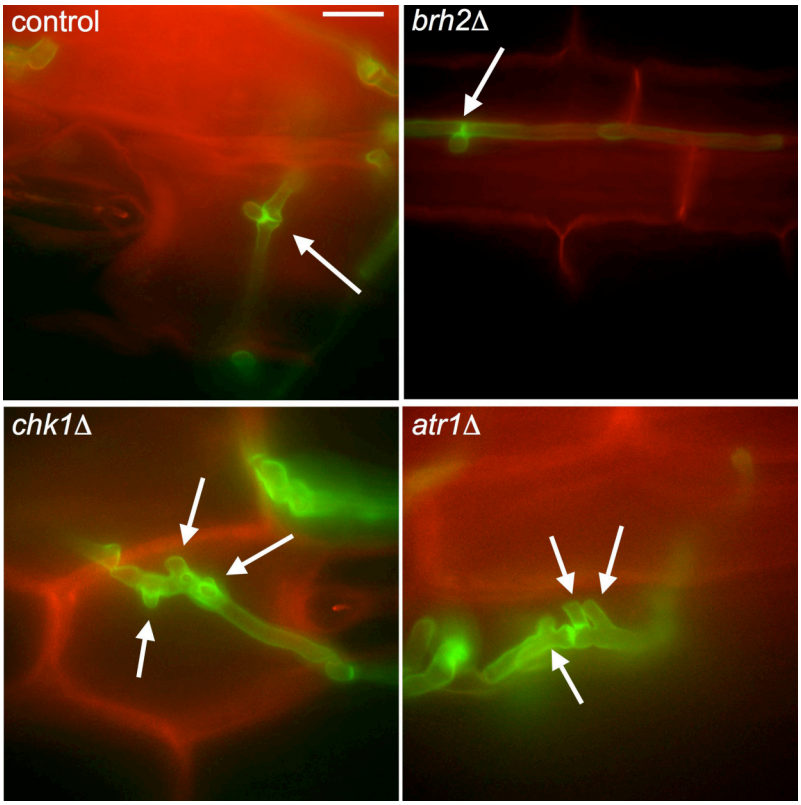
Figure 36: Shoot-like structures observed in the *chk1Δ* and the *atr1Δ* infections

5. Atr1 and Chk1 are required for normal dikaryotic growth *in planta*

The shoot-like structures observed in the tumors had previously been reported in plants infected with *U. maydis* mutants exhibiting an over-activation of the cAMP cascade, and had been attributed to a defective fungal development inside the plant (Kruger et al., 2000). This defect in the development inside the plant was suggested in the Chlorale Black E staining (Fig. 35B), as in *atr1Δ* infection very few hyphae could be seen inside the plant, and they seemed to develop poorly. Therefore a more detailed observation of the fungal proliferation inside the plant was done. For this, infected plant tissue was stained with Alexa-Fluor-labeled wheat germ agglutinin (WGA-AF488), a lectin that binds to chitin, allowing detection of fungal cell walls, and with propidium iodide to visualize plant membranes (Doehlemann et al., 2009). Inside the plant, *U. maydis* grows as a dikaryon. Previous work had reported the formation of clamp-like structures involved in the correct distribution of the nuclei in the hyphae and thereby necessary for *U. maydis* ability to proliferate inside the plant (Scherer et al., 2006). As it can be seen in figure 37A and consistently with this report, wild-type infections as well as *brh2Δ* ones showed hyphae where clamp-like structures could only be seen above of the place where the septum was formed. In contrast with this observation but in agreement with the hypothesis of an impaired fungal proliferation *in planta*, the *chk1Δ* or *atr1Δ* infections showed an aberrant formation and distribution of clamp-like structures (Fig. 37B).

To quantify these defects, hyphae were counted and sorted in relation to the number of clamp-like structures observed at the three more apical septa. Hyphae found in wild-type and *brh2Δ* infections showed the normal distribution of one clamp per septum, meanwhile most of the hyphae from the *chk1Δ* and *atr1Δ* infections showed an aberrant pattern. Cells with none or more than one of these structures per septum were clearly more abundant than normal ones (Fig. 38).

A



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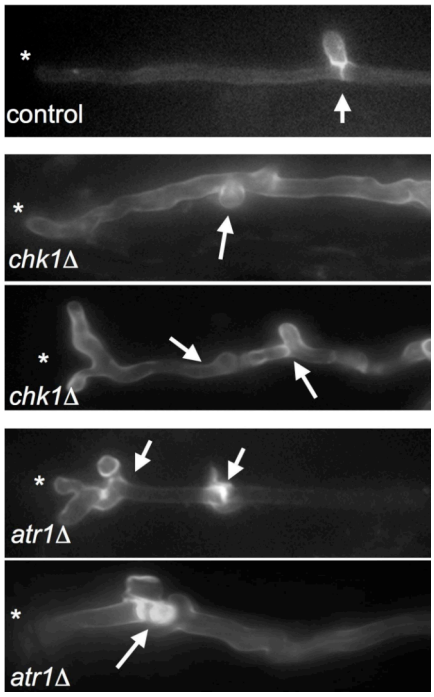


Figure 37: A) Two days post-infection maize plants. Hyphae were stained with WGA-AF488 (shown in green) and plant tissue with propidium iodide (shown in red). Arrows mark the clamp connections. Bar = 10µm. B) Examples of aberrant hyphae morphologies found in *chk1Δ* and *atr1Δ* infections. Asterisks mark the hyphal tip and arrows denote aberrant clamp-like structures.

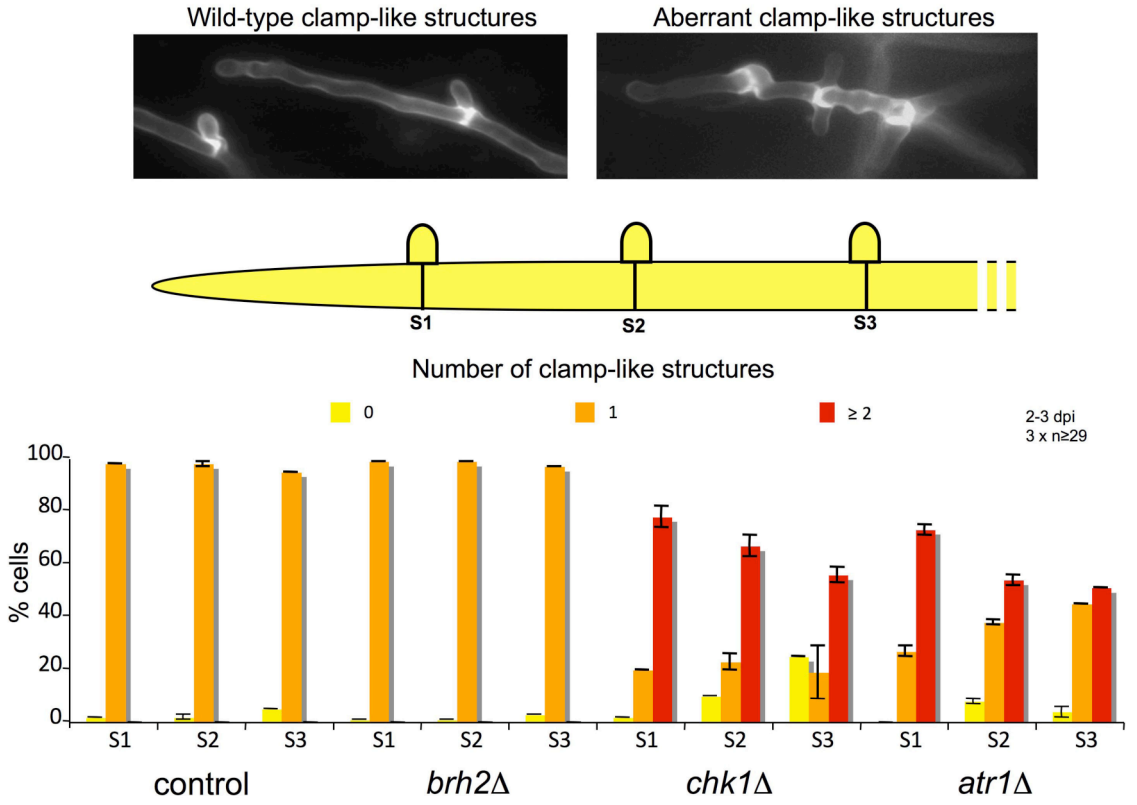


Figure 38: Clamp-like structures quantification in the different infections. Maize plants were stained 2-3 days after infection and clamp-like structures were counted and sorted. The experiment was repeated three times and more than 29 filaments were counted in each one.

Given that clamp-like structure formation is directly related to the process of nuclear division, an analysis of the nuclear distribution was also performed to check whether this process was also affected in the *chk1Δ* and *atr1Δ* strains. To visualize fungal nuclei on infected plants, the strains used in this assay carried a triple GFP gene fused to a nuclear localization signal under the control of the constitutive promoter P_{tef}. While all the wild-type and *brh2Δ* counted hyphae showed two nuclei per cell compartment, in *chk1Δ* and *atr1Δ* hyphae the nuclear content of each cell could vary from one up to four (Fig. 39). These observations suggested that Chk1 and Atr1 had an important role in the dikaryon mitosis and that this impaired nuclear segregation coupled with a wrong septum establishment could lead to a defect in the proliferation of the fungus.

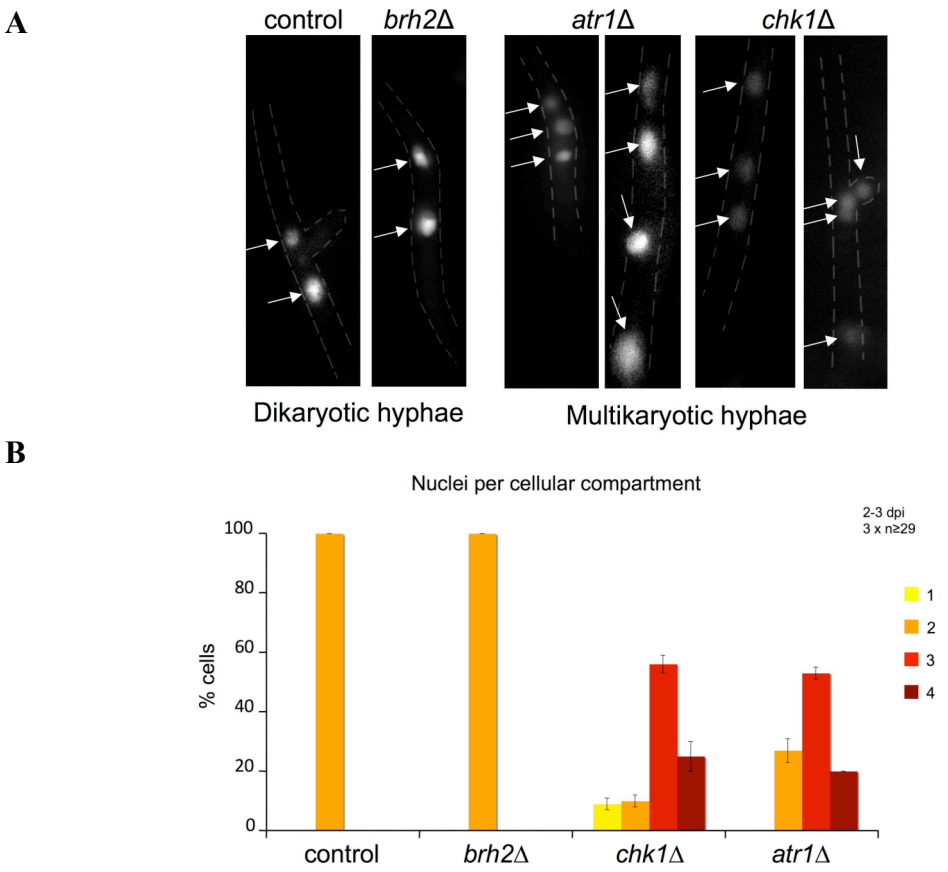


Figure 39: A) Examples of normal dikaryotic hyphae (wild type and *brh2Δ*) and what here is called multikaryotic hyphae as a result of an aberrant nuclear distribution (chk1*Δ* and *atr1Δ*). Used strains in the plant infections were UMP196 x FB2 (control), UMP197 x UCM575 (*brh2Δ*), UMP199 x UMP129 (*chk1Δ*) and UMP198 x UCS6 (*atr1Δ*). B) Quantification of the nuclear distribution on each infection.

6. Atr1 and Chk1 are essential for a correct mitosis in the dikaryon in *Coprinopsis cinerea*

Chk1 and Atr1, as seen by their deletion effects in *U. maydis*, seemed to be essential for a proper regulation of the dikaryon cell cycle. A possible role of the b-homeodomain heterodimer complex in the Atr1-Chk1 axis regulation had been proposed during the infective filament formation. But a question had arisen, Could this b-regulated pathway be conserved in other basidiomycetes? To answer this question the ink cap mushroom, *Coprinopsis cinerea* was used. *C. cinerea* is a well-known model organism where the formation of the dikaryotic mycelium is necessary for the formation of the fruiting bodies. Dikaryon formation has been broadly characterized and it is known to depend mainly on *A* homeoproteins, which are very similar to *U. maydis* b-complex (Casselton and Olesnicky, 1998). *C. cinerea* advantages towards *U. maydis* in

the study of the dikaryon formation were the main reason in using this system. *U. maydis* dikaryon can only be observed inside the plant, which requires a challenging *in planta* microscopic analysis, while in *C. cinerea* this process occurs on a Petri dish surface.

6.1. Atr1 and Chk1 have a role in DNA damage response in *C. cinerea*

A homology search of the main protein kinases involved in the DNA damage checkpoint pathway through the *C. cinerea* genome database website was done. Using the BLAST program on the Broad Institute *C. cinerea* annotated genome webpage (http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/MultiHome.html) and entering umAtr1, umAtm1, umChk1 (*U. maydis* Atr1, Atm1 and Chk1 homologues respectively), scRad53 and spMek1 (*S. cerevisiae* and *S. pombe* Chk2 homologues) as queries, resulted in the identification of several ORFS. An Atr1 homologue located in chromosome 5 and named CC1G_08126.3, an Atm1 homologue situated on chromosome 7 and named CC1G_00839.3, a Chk1 homologue on chromosome 1 named as CC1G_02812.3 and a Chk2 homologue on chromosome 3 and identified as CC1G_09319.3 were found. Subsequently a phylogenetic analysis was done, showing that these proteins grouped together with other family members described (Fig. 40).

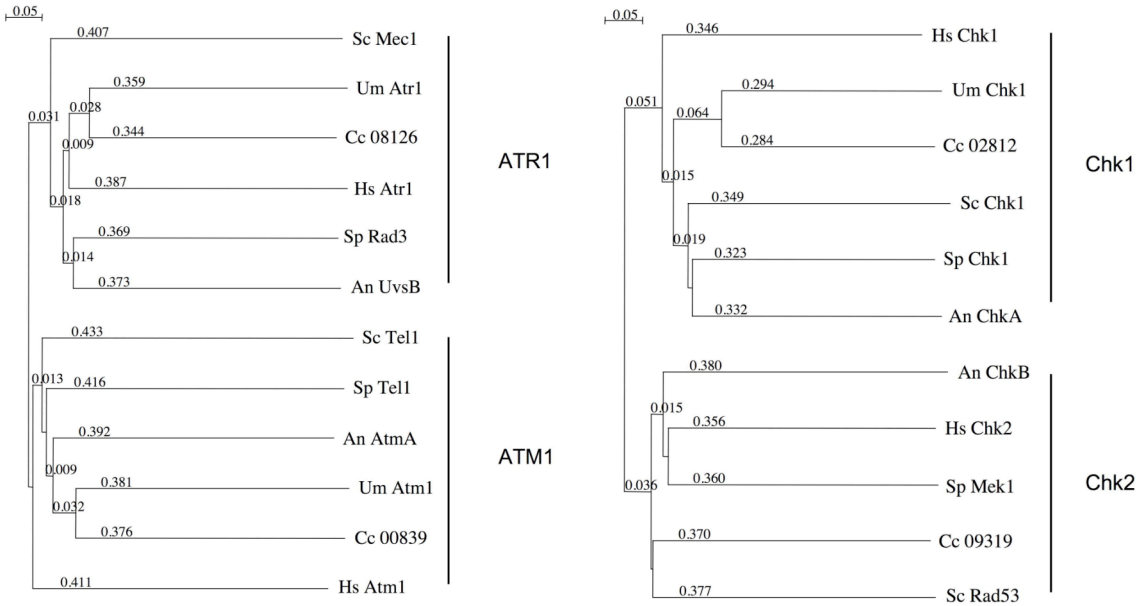
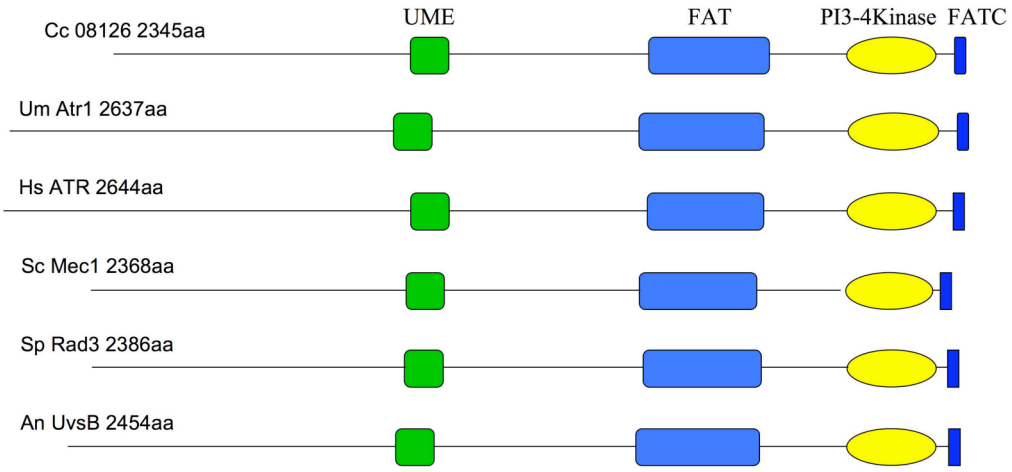


Figure 40: Dendogram obtained from the ClustalW2 program, done by the multiple alignment of different ATR, ATM, Chk1 and Chk2 proteins. Sequences used in the analysis came from *S. cerevisiae*, *U. maydis*, *H. sapiens*, *S. pombe* and *A. nidulans*.

Given that in *U. maydis* we have focused on Atr1 and Chk1, firstly because of their role in the regulation of cell cycle progression and lately due to their effect on the dikaryon formation, we decided to start with the characterization of the putative homologues of these proteins in *C. cinerea*. CC1G_08126 is a 26-exons gene that codes for a protein of around 2350 amino acids and a molecular weight of 264kDa. All the domains that identified this protein family, which were described before for the Atr1 identification in *U. maydis*, could be found here too (Fig. 41A). *cc02812* is a 12-exon ORF that codes for a protein of around 460 amino acids and a molecular weight of 52kDa. Chk1-like proteins are composed of a highly conserved N-terminal kinase domain, a flexible linker region and a less conserved C-terminal region with undefined function (Fig. 41B) (Chen et al., 2000). Due to the similarity of these proteins towards other Atr1 and Chk1 proteins, we decided to designate them from now on as Atr1 and Chk1 respectively.

A



B

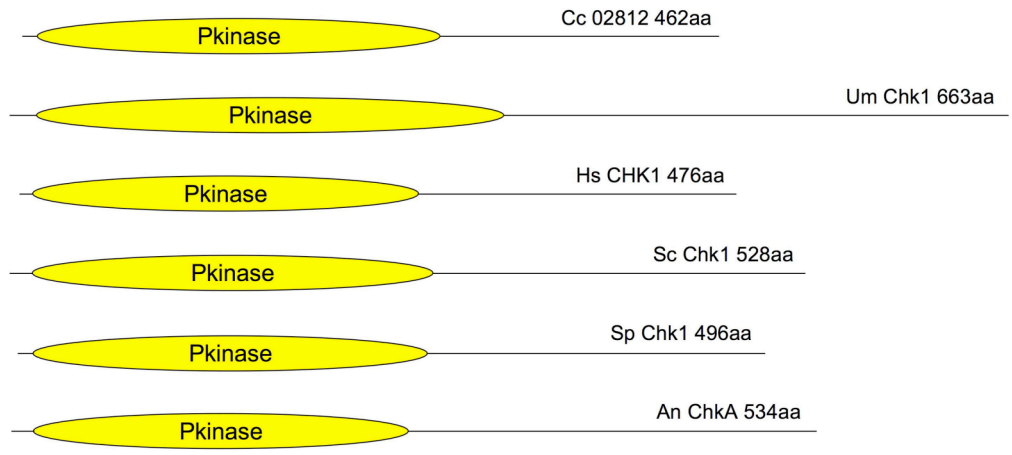


Figure 41: A) Scheme showing the different Atr domains that are also present in *C. cinerea*. B) Scheme for the Chk1 protein family.

Since targeting gene replacement by homologous integration in *C. cinerea* is very rare (only occurs at a frequency of 3-5%) (Binninger et al., 1987), different methods to accomplish the challenging gene targeting have been proposed (Granado et al., 1997; Heneghan et al., 2007; Nakazawa et al., 2011), being one of them RNAi silencing. To study Atr1 and Chk1 activity in *C. cinerea* a silencing strategy was chosen. In a study comparing the different gene silencing methods, Heneghan et al (2007) had reported that the best silencing results were obtained with antisense and hairpin constructs compared to untranslatable ones. And between these two, the higher silencing effect was obtained with the antisense one. Therefore an antisense and a hairpin constructs were designed from different *atr1* or *chk1* exons, and the four resulting plasmids were used to transform *AmutBmut* strain. *AmutBmut* is a self-compatible strain, which means that it can mate with itself due to the mutations in both mating loci (Swamy, 1984). As selective marker *pab1* gene was used. The structures and the cloning procedures of these plasmids are detailed in materials and methods in figures 5 to 7. At the same time, *AmutBmut* strain was also transformed with the empty plasmid used for the silencing as a control (RNAi control). From each transformation, around 50 colonies were isolated and their ability to grow on minimal media, which only occurs in those cells that had inserted the *pab1* gene and therefore the silencing construct in their genome, was checked. From all the transformants, 18 colonies from the *atr1* silencing, 14 colonies from the *chk1* silencing and 8 from the RNAi control were analyzed by RT-PCR. RNA from fully-grown plates was isolated and retro-transcribed to cDNA before performing a relative quantitative PCR. As housekeeping, *benA* gene, that codes for the β-tubulin was used (Walti et al., 2006). Oligos used in this assay are listed in table 5 and their efficiency is detailed in table 4. Finally, two clones silenced in *atr1*, two in *chk1*, with a different silencing degree and one clone carrying the control construct were chosen. In the *atr1* silenced clones, RNAi *atr1*#1 was silenced on a 30%, meanwhile RNAi *atr1*#2 was affected on an 80%. In the *chk1* clones, RNAi *chk1*#1 was silenced in a 30% and RNAi *chk1*#2 on a 50% (Fig. 42). In both gene silencing the selected clones came from the antisense construct transformants, which seemed to work better than the hairpin one, as it had been previously observed (Heneghan et al., 2007).

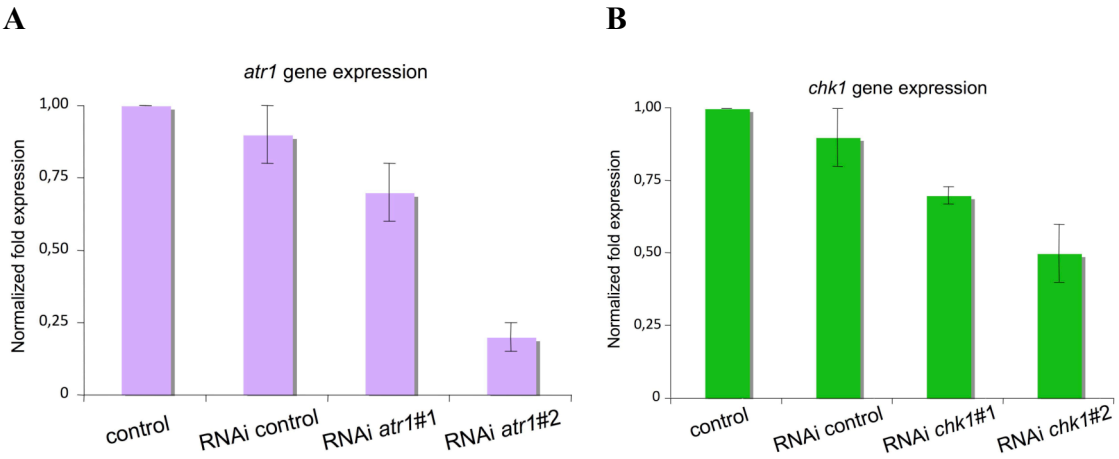


Figure 42: Relative expression of *atr1* and *chk1* in the selected *C. cinerea* silenced clones.

As we had seen in *U. maydis* and it had previously been described in other model organisms, Atr1 deficiency make cells more sensitive to genotoxic agents, specially to HU, which causes DNA replication stress (Koc et al., 2004). It is because of this that the sensitivity against genotoxic agents of the selected colonies was tested. HU and MMS were used and the results are shown in figure 43. For this, an equal size mycelium fragment was inoculated on rich plates supplemented with different genotoxic substances. The strains were grown until the plate was fully covered in the control conditions. For the control strains, 6 days were needed until the pictures could be taken, meanwhile for RNAi *atr1*#1 and RNAi *atr1*#2 8 days and 10 days, respectively, were necessary and for RNAi *chk1*#1 and RNAi *chk1*#2 it took 7 days. This indicated that the normal growth rate was affected.

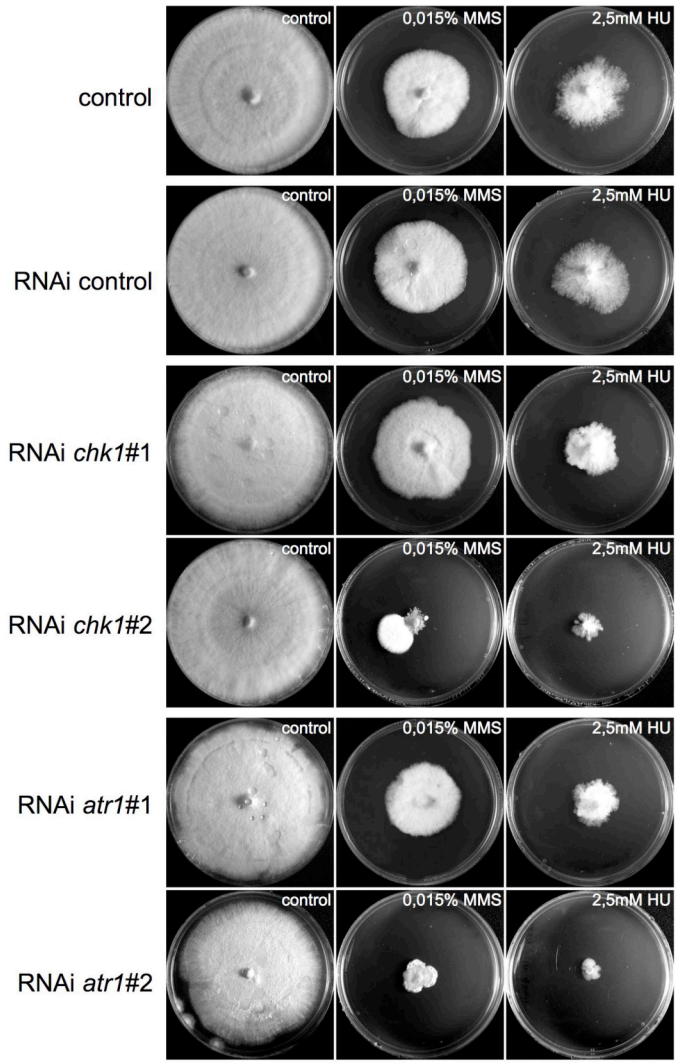


Figure 43: *atr1*-silencing effect towards DNA damage in *C. cinerea*.

Taking all these results together we could see that Atr1 and Chk1 have a role in the DNA damage response pathway in *C. cinerea*, and even more that the difference in silencing observed by the quantitative PCR analysis correlates with the sensitivity degree shown by the strains. RNAi *atr1*#1 and RNAi *chk1*#1 are less silenced than RNAi *atr1*#2 and RNAi *chk1*#2.

6.2. Atr1 and Chk1 role in dikaryon in *C. cinerea*

It had previously been described that the formation of a fertile dikaryon in *C. cinerea* relies in the compatibility of its two mating loci *A* and *B* (Raper, 1953), and that the *A* locus is homologous to the *b* mating type of *U. maydis*. The *A* locus controls several processes essential for the dikaryon formation which are the pairing of nuclei, the initiation of clamp cell formation, the synchronized nuclear division and the septation (Kues, 2000). To check if the Atr1-Chk1 axis had a role in these *A*-regulated

processes, the nuclear distribution and the clamp cell formation in the strain *AmutBmut* carrying silencing constructs for *chk1* or *atr1* was analyzed. The fungus was grown on a thin agar layer upon a microscope glass slide for 2 days. After, it was stained with Hoechst, a blue fluorescent dye that binds double stranded DNA and calcofluor-white another fluorescent that binds to structures containing cellulose and chitin (Virag et al., 2007). The obtained results reminded very much to those previously seen in *U. maydis*. As control strains a wild-type *AmutBmut* and the *AmutBmut* transformed with the empty plasmid used for the silencing were used. In these, all the observed hyphae showed one or two nuclei per cell, meanwhile in the *chk1* and *atr1* silenced strains cells with more than two nuclei or nuclei "trapped" in the clamps could be observed (Fig. 44).

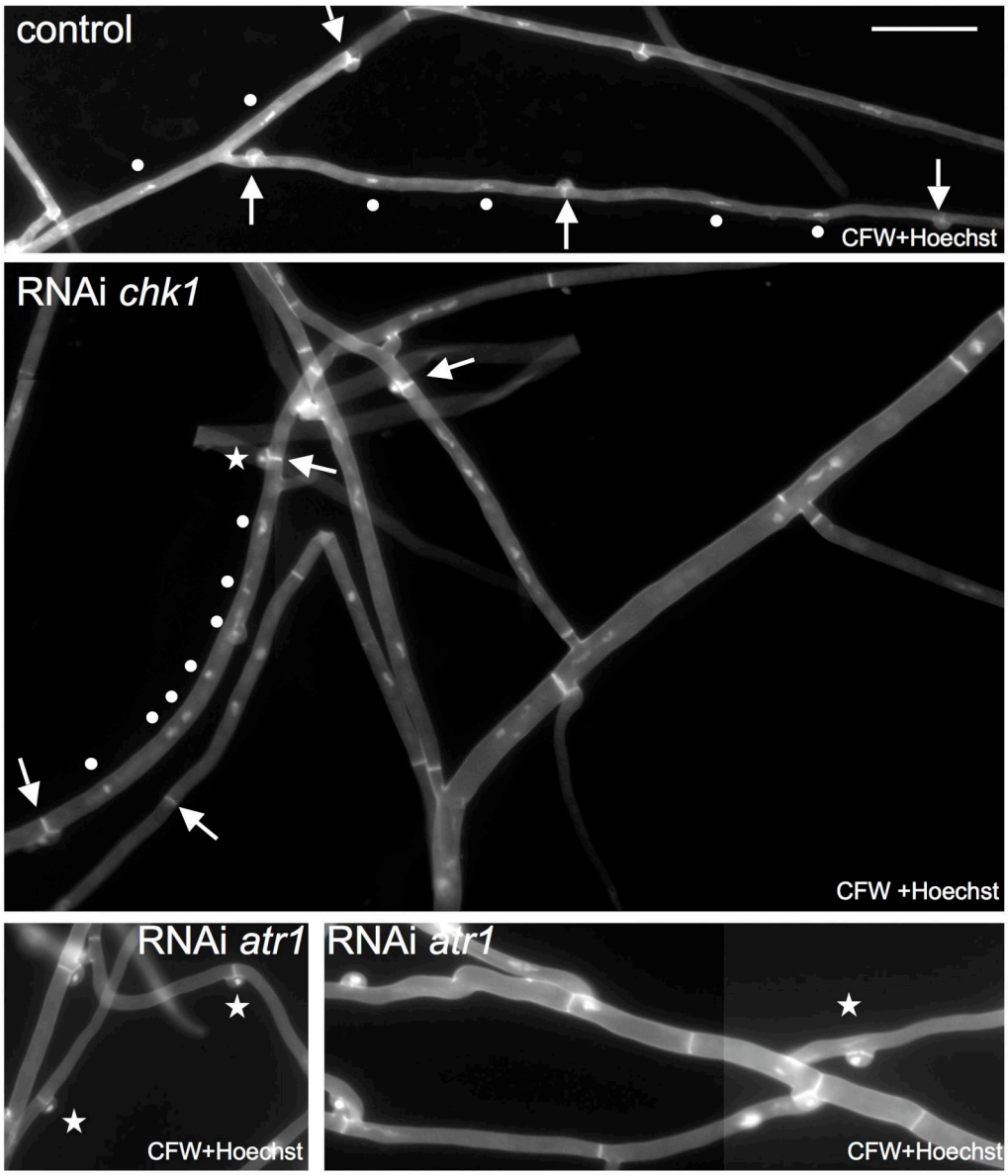


Figure 44: Hyphae from control and silenced strains. Arrows denote septum; circles mark the nuclei and the stars show nuclei locked in clamp cells. Bar = 15μm

To quantify the abnormalities observed in the *chk1* and *atr1* silenced strains; a quantification of the number of nuclei per cellular compartment as well as the presence of normal or aberrant clamps in each of these cells was done. Graphic shown in figure 45 denotes the results obtained, where "wt mitosis" gather all the cells where the presence of nuclei and clamps was normal, "aberrant mitosis" groups those cells where either the nuclear distribution or the clamp formation was aberrant and "trapped nuclei" shows the nuclei that were observed "locked" inside the clamps.

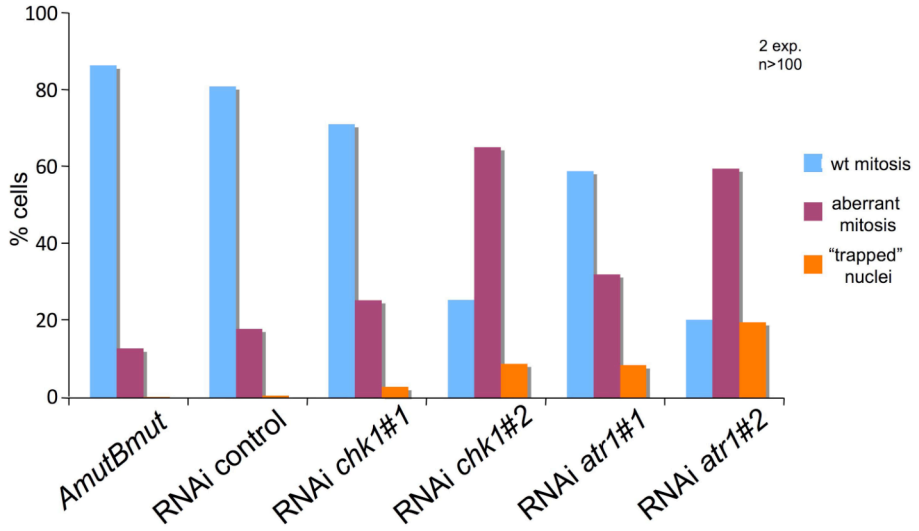


Figure 45: *chk1* and *atr1* silencing in *C. cinerea* mycelium induces the appearance of aberrant cells. Samples were grown on a thin layer of rich medium over a microscope glass slide for 2-3 days.

In the control strains the relationship between uninucleated and binucleated cells was similar to previously observed results (Polak et al., 1997) and the number of aberrant phenotypes was very low, but in our silenced strains this number increased. In the less silenced strains (RNAi *chk1*#1 and RNAi *atr1*#1) the total aberrant phenotypes represented a 30-40% of the cell population, meanwhile in the most silenced ones (RNAi *chk1*#1 and RNAi *atr1*#1) the aberrant cell population raised up to a 70-80%. So accordingly with these results and the results obtained in *U. maydis* it seemed that the homeodomain proteins induced the Atr1-Chk1 axis in an essential way for the correct regulation of the dikaryon cell cycle.

7. Atr1 and Chk1 are necessary for mature fruiting body formation in *C. cinerea*

Fruiting-body formation is the most complex developmental process in the life cycle of *C. cinerea* that starting from a mesh of free an undifferentiated hyphae ends up in a compact structure with differentiated tissues inside (Moore, 1995). The production of fruit bodies in most higher basidiomycetes depends on the formation of the dikaryotic mycelium and the correct environmental conditions (Stahl and Esser, 1976). These environmental conditions including temperature, humidity, light and nutrients play an important role in determining the developmental pathway that *C. cinerea* follows. And in the fruiting body initiation the first three factors are crucial (Kues, 2000). Since it had been seen that the silenced strains were impaired in the correct formation of the dikaryon, we wondered what would happen when the fruiting body program was induced. Firstly, the formation of mushrooms was evaluated under nutrient-controlled conditions. For this, each strain was grown on 15 YMGT plates until the mycelium filled up all the plate surface and just before the hyphae reached the border, the growing conditions of these plates were changed from 37ºC and 24 hours dark to 30ºC, >90% humidity and a 12 hours light/12 hours dark regime. This experiment was done three times with similar results. In the control strains, all the different developmental stages that had been previously described were observed (Navarro-Gonzalez, 2008). Hyphal knots that became primordia and finally gave rise to mature fruiting bodies were developed normally. But in the silenced strains no fully developed mushrooms could be observed. Both *atr1*-silenced strains could only form hyphal knots. In the *chk1* silenced strains, interestingly RNAi *chk1*#1 presented only hyphal knots, meanwhile in RNAi *chk1*#2, which is the most silenced, early stage primordia that aborted before becoming a mature primordia could be observed on a 70% of the plates (Fig. 46 and 47).

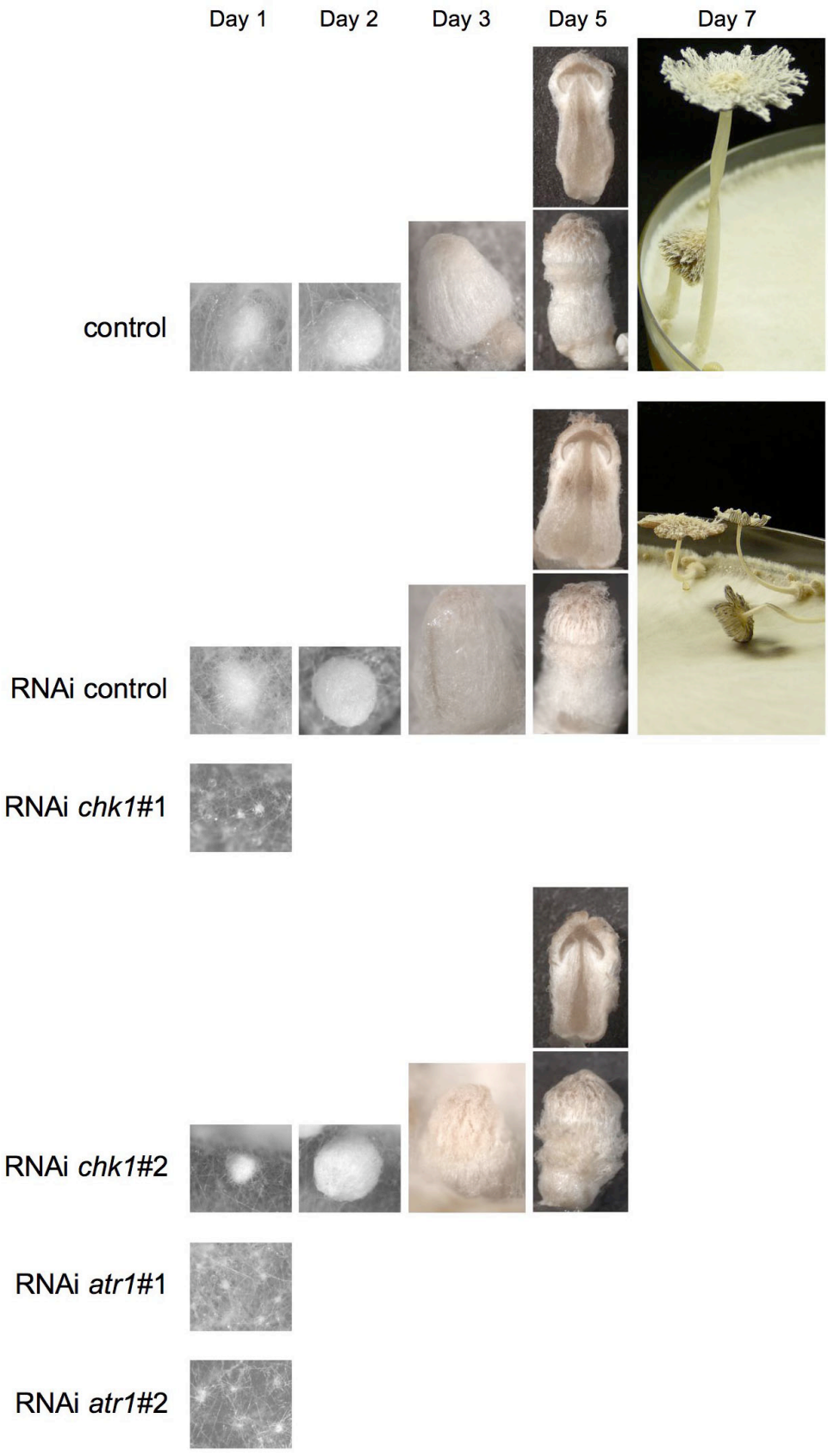


Figure 46: Silencing of *chk1* and *atr1* avoids the formation of mature fruiting bodies in *C. cinerea*.

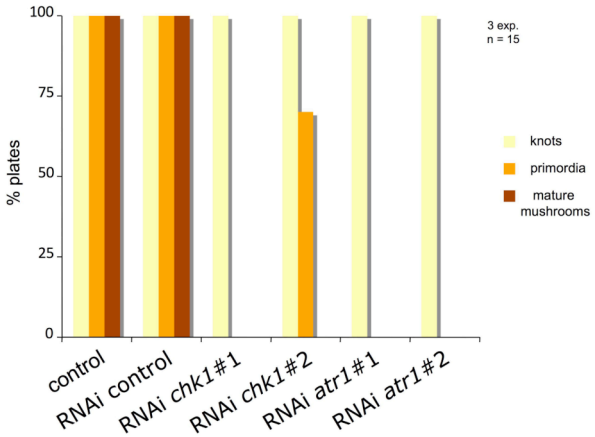


Figure 47: Quantification of *chk1* and *atr1* silencing effects on the different developmental stages of the fruiting body process.

Afterwards the nutrient conditions were changed to see if this factor could also affect the fruiting of the strains. This was done by using horse dung, the natural substrate of this species (Buller, 1931). For this, fresh horse dung was autoclaved to sterilize it and subsequently each strain was inoculated in 6 flasks that contained the aforementioned media. The strains were grown until they colonized the entire medium and then their growing conditions were changed as described before. The results obtained in this experiment differed from the ones obtained in the plates. In this case mature fruiting bodies were observed not only in the control strains but also in RNAi *chk1*#1 and RNAi *atr1*#1, which are the less silenced strains. The number an distribution of the mushrooms was not the same, in the control strains there were about 10 mature fruiting bodies and were distributed around the whole flask, meanwhile in the silenced strains the number of mushrooms per bottle was about 4 and they were located on a certain place. In RNAi *chk1*#2, again primordia that arrested their development before reaching the mature state were observed and in RNAi *atr1*#2 only very few hyphal knots were formed (Fig. 48).

The differences in the media composition clearly affected the fruiting of the *chk1* and *atr1* less silenced strains. But in the most silenced strains the effect of this change in the media composition was not enough to induce the formation of mature mushrooms. This indicated that the processes controlled by these genes seemed to be essential for the completion of *C. cinerea* life cycle and that their role might be conserved in other Basidiomycetes due to the resemblance of the effects previously seen in *U. maydis*.

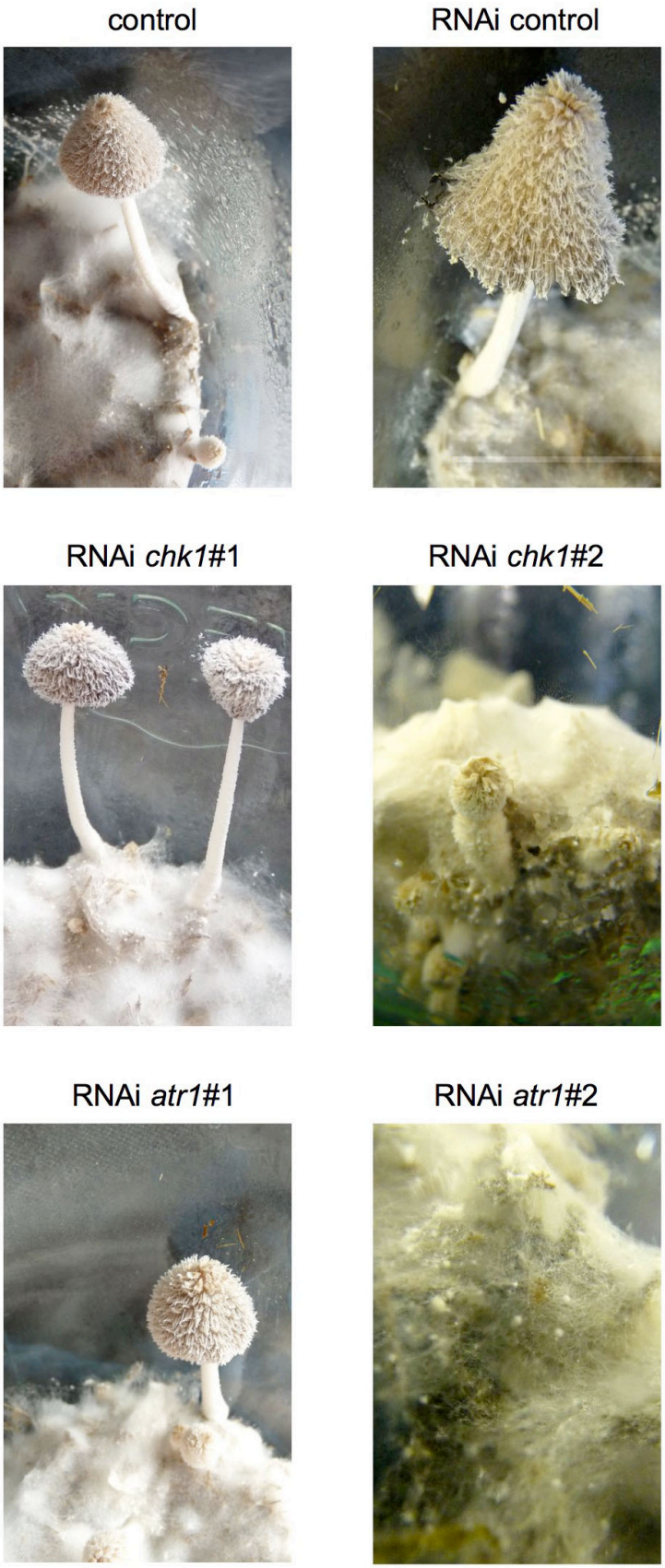


Figure 48: Fruiting body formation could be induced in RNAi *chk1*#1 and RNAi *atr1*#1 by changing the media composition.

Atr1 and Chk1 in telomere damage signaling in *Ustilago maydis*

1. Uku70/80 characterization in *U. maydis*

1.1. Uku70 and Uku80 identification in *U. maydis*

Ku70 and Ku80 *U. maydis* homologues were identify through a homology search done by the BLAST program. Yku70 and Pku70 or Yku80 and Pku80, Ku70 and Ku80 homologues in *S. cerevisiae* and *S. pombe* respectively were used as queries. The search was done on the Munich Information Center for Protein Sequences *U. maydis* database and subsequently the results were confirmed by performing a BLAST on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of these searches were two loci that had previously been manually annotated as *um05148* and *um05756*. A phylogenetic analysis of these sequences included them within the Ku70 and Ku80 like proteins respectively (Fig. 49).

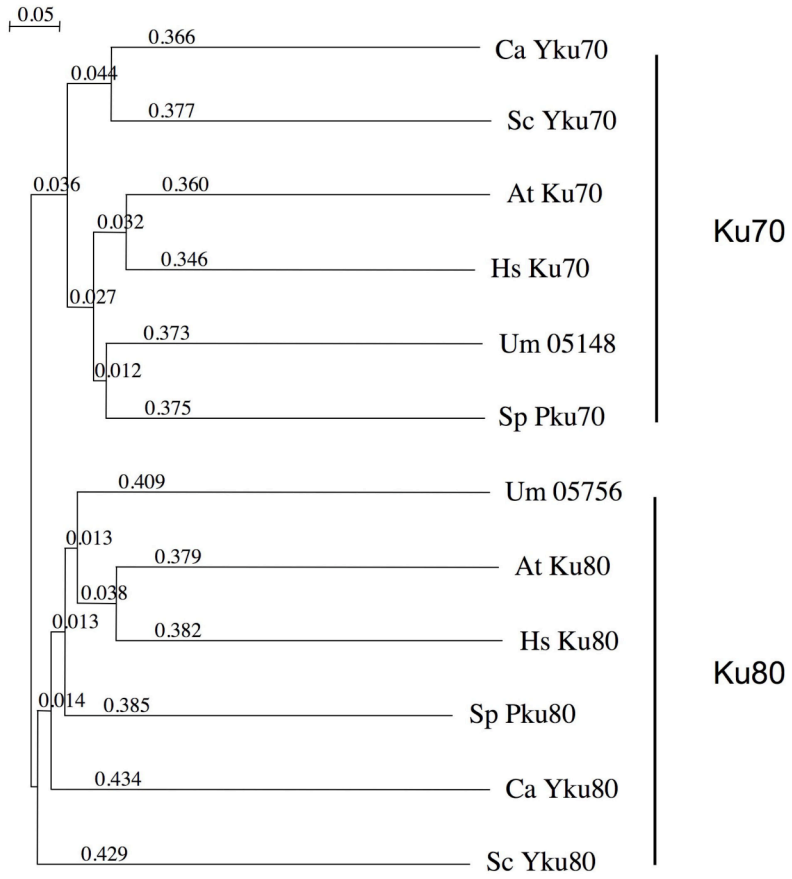
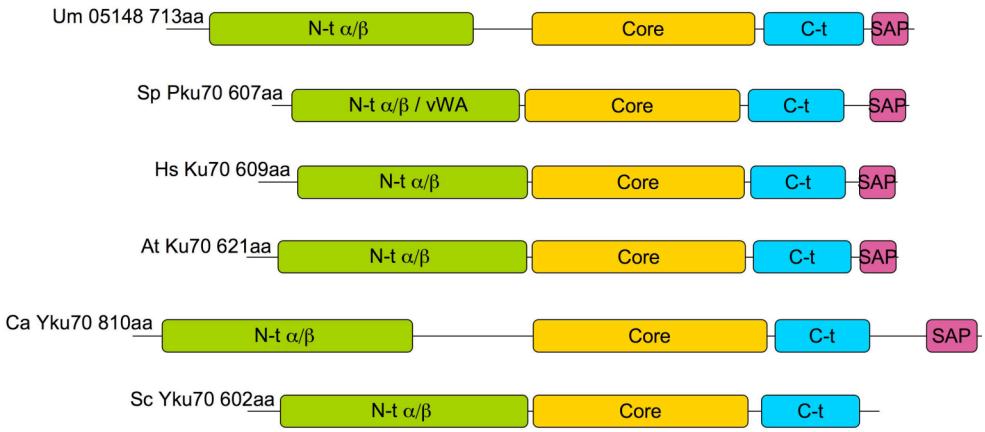


Figure 49: Dendogram of Ku-like proteins obtained from the ClustaW2 program. Ku-like sequences used in the analysis came from *Arabidopsis thaliana*, *Homo sapiens*, *Candida albicans*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*.

Um05148 coded for a 713 amino acids protein with a molecular weight of around 80kDa, which grouped within the Ku70-like proteins. And Um05756 coded for a protein of 826 amino acids and a molecular weight of around 92kDa, which was put together with the Ku80-like proteins. Ku70 and Ku80 share a common topology and form a coupled-pseudosymmetrical molecule with a ring inside that encircles duplex DNA. Ku70 and Ku80 are formed of three shared domains and a divergent C-terminal domain. The shared domains are a N-terminal α/β domain or von Willebrand A domain that is thought to be a protein-protein interaction site with a role in binding to other repair factors, a central core formed of β-barrel domains with a DNA binding activity dependent on the heterodimer formation and a helical C-terminal arm which embraces the opposite subunit core domain (Walker et al., 2001; Wang et al., 1998). In some eukaryotes, Ku70 presents a SAP domain at the carboxy-terminus, named after three proteins containing this motif (SAF-A/B, Acinus and PIAS) and which has been described as a DNA binding domain (Aravind and Koonin, 2000). This domain activity has been shown in Ku70 to be dispensable for the heterodimer DNA binding activity and a role in pausing Ku at specific DNA sequences has been proposed. In Ku80 at the carboxy-terminus, sometimes there is an extension that is thought to bind the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Harris et al., 2004).

As it can be seen in figure 50, all the described motifs were present in Um05148 and Um05756, therefore it was agreed to designate them as Uku70 and Uku80 each to each.

A



B

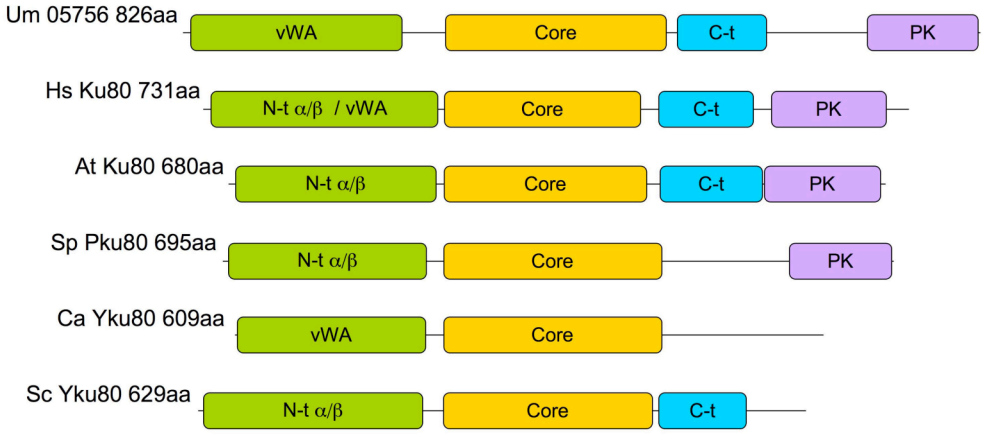


Figure 50: Schematic representation of several Ku70-like proteins (A) and Ku80-like proteins (B) showing the characteristic domains. Data were obtained from the InterProScan tool at the EMBL-EBI website (**http://www.ebi.ac.uk/interpro/**).

1.2. Uku70 and Uku80 are essential in *U. maydis*

Ku heterodimer is best known for its roles in DNA repair through non-homologous end-joining (NHEJ). In response to double strand breaks, Ku binds directly to DNA avoiding these from drifting apart or protecting them from unwanted nucleolytic attacks. In eukaryotic cells an important role for Ku in telomere maintenance has also been described (Downs and Jackson, 2004). Its roles at telomeres are several, including telomere protection and recruitment or stabilization of telomerase. In fact, in humans, Ku heterodimer has been described to be essential and this essentiality has been associated to its role in telomeres. Previous attempts in *U. maydis* had suggested that *uku70* and *uku80* might be essential (W.K. Holloman personal communication), therefore a strategy to create conditional strains in which the gene expression could be controlled was designed. For this, the inducible promoter P_{nar1} was used in the substitution of both endogenous promoters (Fig. 51).

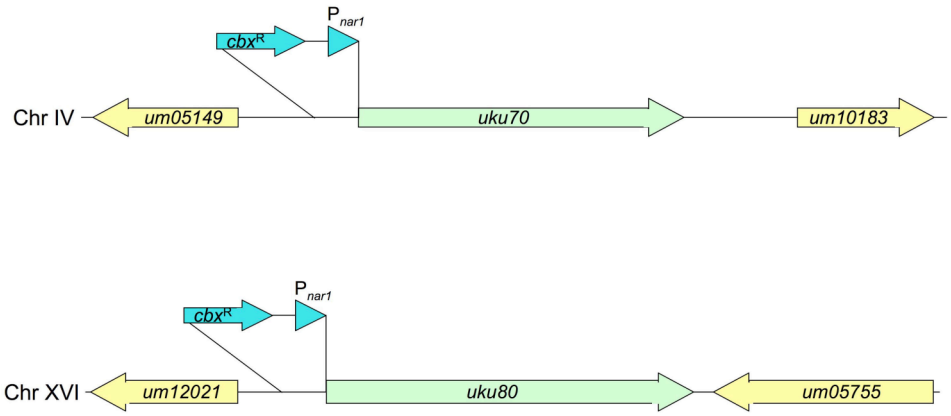


Figure 51: Scheme showing the promoter replacement carried out in both loci.

Thanks to this change, gene expression could be easily controlled by changing the growth media. As we have previously seen, P_{nar1} gets active in presence of NO_3 and in rich media is repressed. This promoter change was verified by RT-PCR (oligos are listed in Table 5 and their efficiencies are detailed in Table 4). As it appears in figure 52A, when the strains were grown on $MMNO_3$ *uku70* and *uku80* expression was around 7 times the wild type, this is because in NO_3 P_{nar1} is a strong promoter, but when the strains were transferred to rich media there was no expression of these genes. After this, serial dilutions were done to observe the *uku70* and *uku80* absence effect on the cells. As it is shown in figure 52B, *uku70* and *uku80* seemed to be essential in *U. maydis*, as the cells lacking one of the genes could hardly grow. Also *uku70* and *uku80* high expression levels in NO_3 did not seem to affect the cells.

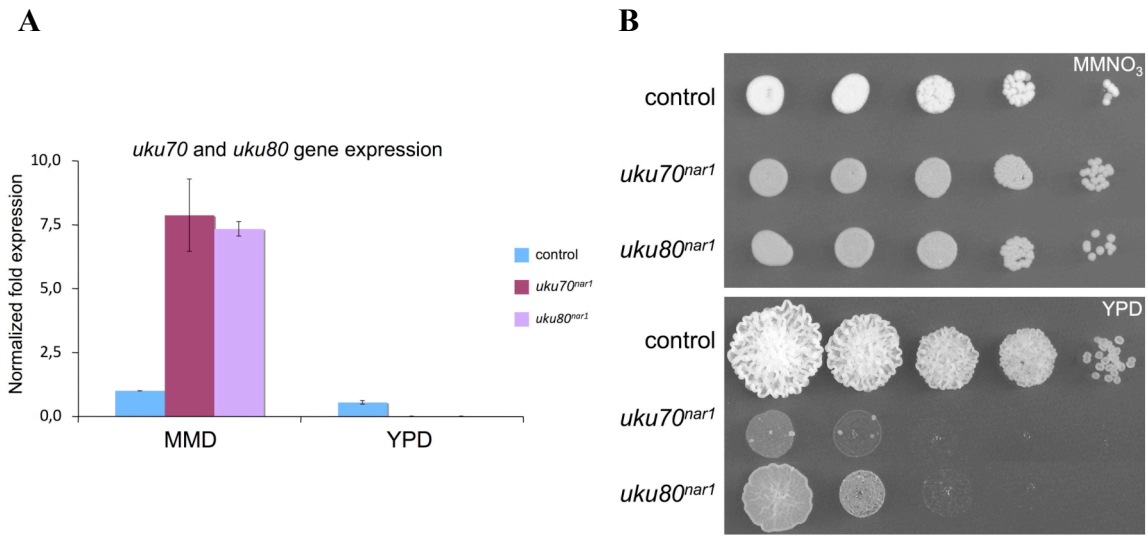


Figure 52: *uku70* and *uku80* are essential in *U. maydis*. A) Results from the relative quantitative PCR. Data were normalized to the *α–tubulin* and the wild type strain by the Pfaffl method. B) Serial dilutions of the control (FB1), $uku70^{nar1}$ (UCS33) and $uku80^{nar1}$ (UCS30) cells.

2. Uku70/80 deficiency activates the DNA damage checkpoint

We wondered which could be the reason why *uku70* and *uku80* were essential in *U. maydis* hence we decided to analyze the cells. A *cut11-Cherry* allele was inserted in control, in *uku70*^{nar1} and in *uku80*^{nar1} strains. Cut11 is a protein that binds to the nuclear pore complex (homologue to *S. pombe* Cut11) and therefore a well described nuclear membrane marker (Perez-Martin, 2009; West et al., 1998). Given that *U. maydis* undergoes an open mitosis, this marker helps to distinguish G_2 phase from early mitosis (Straube et al., 2005). At the same time the nuclei of the cells were stained with DAPI. In wild type cells and *uku70*^{nar1} or *uku80*^{nar1} cells grown in $MMNO_3$ one or two nuclei per cell, depending whether they had undergone through mitosis or not, could be observed, while in *uku70*^{nar1} and *uku80*^{nar1} cells grown in YPD, all of them presented only one nucleus per cell (Fig. 53).

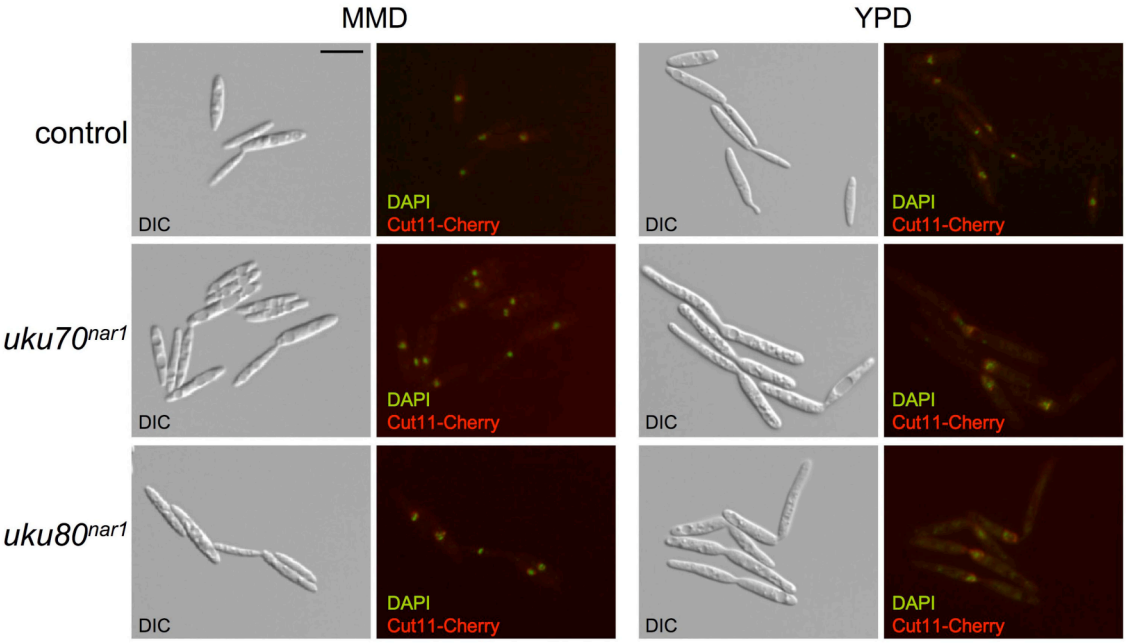


Figure 53: *uku70* and *uku80* absence induce a G_2 cell cycle arrest. Control (UMP132), *uku70*^{nar1} (UCS34) and *uku80*^{nar1} (UCS37) strains carrying a Cut11-Cherry fusion were grown under expressing ($MMNO_3$) and non-expressing *uku* conditions (YPD) overnight and subsequently the nuclei of the cells were stained with DAPI. Bar = 10µm

The presence of cells with elongated buds, a G_2/early mitosis stage hallmark in *U. maydis* (Perez-Martin et al., 2006) together with the observation of only one nucleus per cell with an intact nuclear membrane clearly suggested a G_2 cell cycle arrest. Taking these observations into account an analysis of the cellular DNA content of our *uku70*^{nar1}

and *uku80*^{nar1} strains by flow cytometry was performed. Cell samples were taken on $MMNO_3$ and YPD and the DNA was stained with propidium iodide. As it can be seen in figure 54, *uku70*^{nar1} and *uku80*^{nar1} cells grown on rich media showed a clearly G_2 arrest. Note that the pick in *uku70*^{nar1} cells grown in rich media was bigger than 2C, which could be associated to the accumulation of mitochondrial DNA.

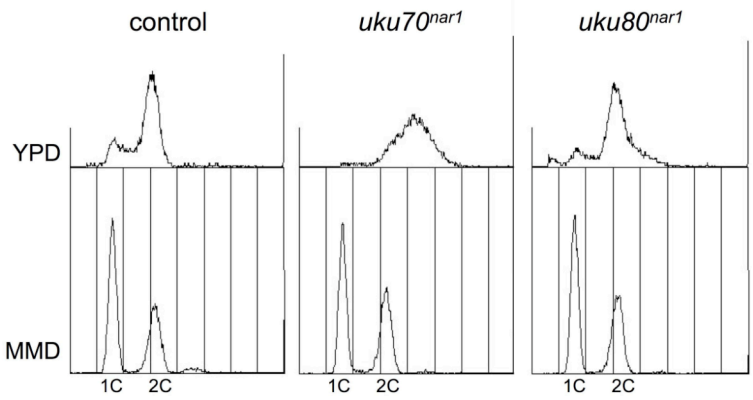


Figure 54: The absences of *uku70* or *uku80* induce a G_2 cell cycle arrest. FACS analysis of the control, *uku70*^{nar1} and *uku80*^{nar1} strains grown under expressing ($MMNO_3$) and non-expressing *uku* conditions (YPD).

All these results suggested a cell cycle arrest in G_2 phase, but could it be the consequence of the activation of the DNA damage response pathway? As we have seen earlier, Checkpoint kinases are the key regulators of the cell cycle progression in response to DNA damage and in *U. maydis* there is only a Chk1 homologue. Therefore we wondered if in this G_2 arrest Chk1 was involved. For this a *chk1-GFP* allele was inserted in *uku70*^{nar1} and *uku80*^{nar1} strains (Fig. 55). Interestingly, when *uku70* and *uku80* expression was turned off and cells showed a G_2 arrest, a clearly nuclear Chk1 localization could be observed. As we have previously seen, the nuclear Chk1 localization is connected with Chk1 activation. In control cells grown in either media and in *uku70*^{nar1} and *uku80*^{nar1} cells grown in $MMNO_3$ no Chk1 nuclear localization could be observed. But in absence of either *uku70* or *uku80* a clear Chk1 nuclear localization was seen.

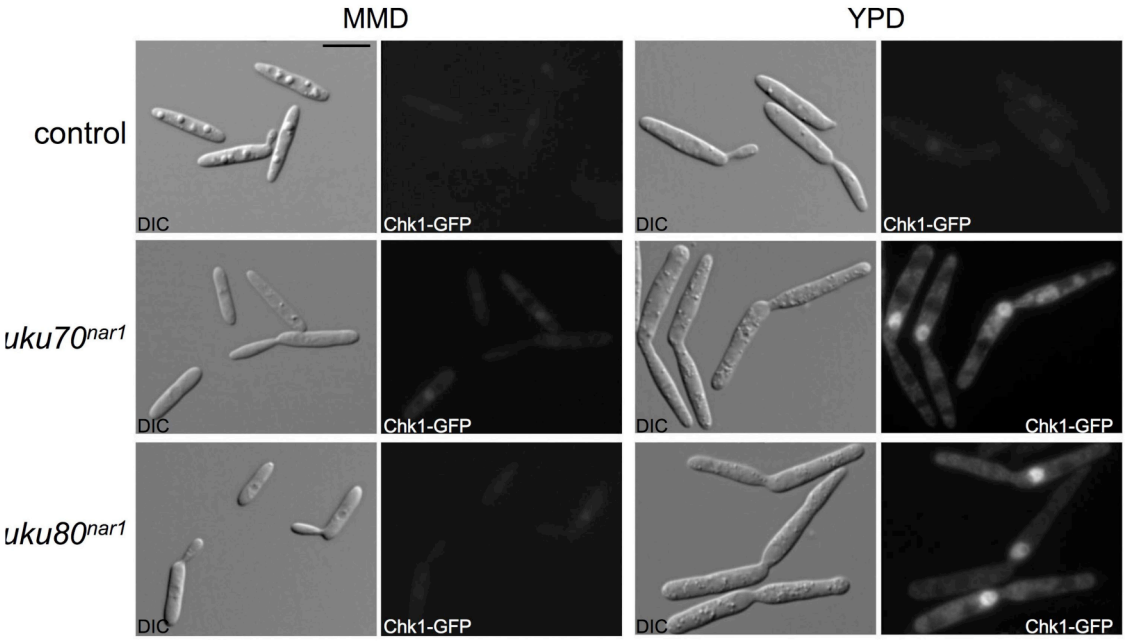


Figure 55: Chk1 is activated in the G_2 cell cycle arrest induced by *uku70* and *uku80* absence. Control, *uku70*^{nar1} and *uku80*^{nar1} strains carrying a *chk1-GFP* allele were grown under expressing ($MMNO_3$) and non-expressing *uku* conditions (YPD). Bar = 10μm

Taking all these results together, it seemed that the down-regulation of *uku70* or *uku80* expression leaded to a cell cycle arrest coupled to an activation of Chk1.

3. *uku70/80* essentiality can be avoided by deleting *atr1* or *chk1* in *U. maydis*

To see if the observed cell cycle arrest was dependent of Chk1, a deletion of the DNA damage response pathway members identified in *U. maydis* was planned. As we have seen in "Atr1 and the DNA damage response pathway in *Ustilago maydis*", the Atr1-Chk1 axis, which has broadly been described in other organisms, was conserved in *U. maydis* and became active in response to DNA damage. Therefore *chk1* and *atr1* were deleted in *uku70*^{nar1} and *uku80*^{nar1} strains. The nuclear DNA content of these strains was again analyzed by flow cytometry, and a growth assay was performed by doing serial dilutions on $MMNO_3$ and YPD media. As it can be observed in figure 56A, the previously observed G_2 cell cycle arrest in *uku70*^{nar1} and in *uku80*^{nar1} cells grown on rich media disappeared when *chk1* or *atr1* were deleted. This rescue could be also seen in the drops assay shown in figures 56B and 56C, although here a difference between the *chk1* deletion effect and the *atr1* one could be noticed. In both *uku*^{nar1} strains, *chk1*

deletion fully recovered the viability of the cells; meanwhile the recovery from *atr1* deletion seemed only to be partial.

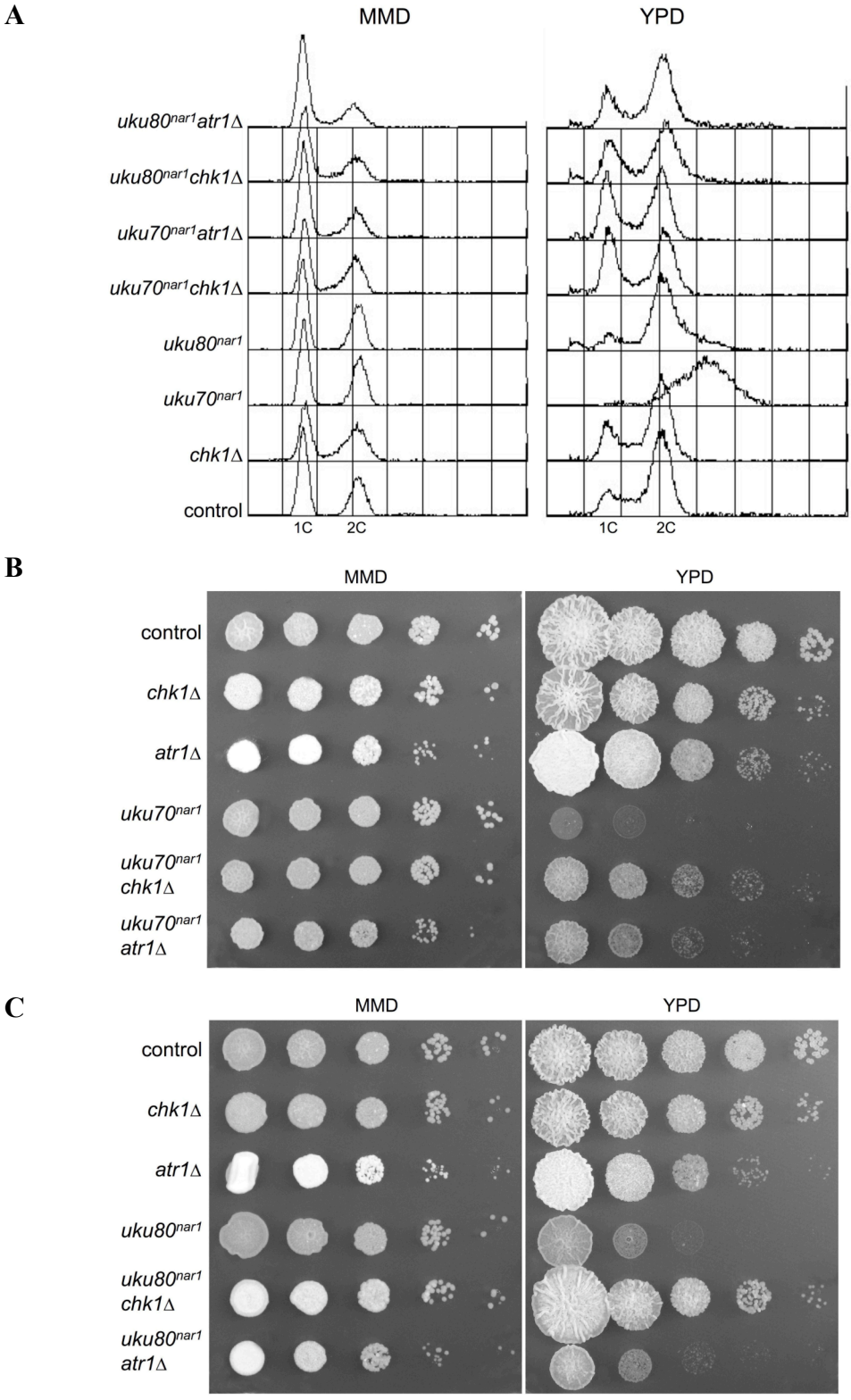


Figure 56: *uku70* and *uku80* essentiality can be rescued by *chk1* or *atr1* deletion. In these analysis the following strains were used: FB1, UMP122, UCS30, UCS33, UCS35, UCS39, UCS40 and UCS44. A) FACS analysis of the DNA content of these strains grown in expressing ($MMNO_3$) and non-expressing conditions (YPD). B and C) Serial dilutions of the strains, showing the rescue of the *uku70* and *uku80* absence lethal phenotype.

What attracted most our attention was the fully viability rescue by *chk1* deletion in *uku70*^{nar1} and *uku80*^{nar1} cells. This showed that the essentiality of these genes was due to checkpoint activation and that no essential processes seemed to be affected upon *uku70* or *uku80* depletion.

4. Uku70/80 depletion activates DNA damage signaling at telomeres

The DNA damage checkpoint activation observed after *uku70* and *uku80* depletion, which was associated to their essentiality, could be due to Ku described roles in NHEJ or in telomere maintenance. Roles in telomeres or NHEJ can be distinguished by the involvement of the DNA ligase IV, which have roles only in NHEJ. For instance, in humans, Ku essentiality has been associated to its role in telomeres due to the presence of viable mutations in genes from the NHEJ pathway like DNA-PKcs or DNA ligase IV, which indicates that this process is not essential (Wang et al., 2009). Ku proteins have been described to play several important roles at telomeres: regulation of telomere addition, protection of telomeres against recombination and nucleolytic degradation, promotion of telomere-proximal genes transcriptional silencing and nuclear positioning of telomeres (Fisher and Zakian, 2005). Alteration of telomere length affects cell survival, therefore a regulation of these structures during the cell cycle is essential (Greider, 1996). We hypothesized that Uku essentiality could be related to telomeres because in *U. maydis ligIVΔ* mutants are viable (W.K. Holloman personal communication). Information about the structure and function of telomeres in filamentous fungi and particularly in *U. maydis* is scarce. All the putative telomerase components have been identify by sequence homology, as well as some of the telosome or shelterin complex associated proteins in *U. maydis* (Sanchez-Alonso and Guzman, 2008). We wondered whether the Chk1 activation observed upon *uku70* or *uku80* deletion in *U. maydis* was due to telomere damage. Therefore a GFP-Rad51 fusion was used as a DNA damage reporter. To know if the Rad51foci were pointing to telomere damage Pot1 homologue was used. Pot1 (Protection Of Telomeres) is a key component required for the protection of chromosome ends and for the regulation of telomere length in *S. pombe* and mammals (Baumann and Cech, 2001). Pot1 binds to single-stranded telomeric DNA. An endogenous Pot1-Cherry fusion and a GFP-Rad51 fusion were inserted into wild type, *uku70*^{nar1}, *uku80*^{nar1}, *uku70*^{nar1}*atr1Δ*, *uku80*^{nar1}*atr1Δ*,

uku70^{nar1}*chk1Δ* and *uku80*^{nar1}*chk1Δ* cells and co-localization of both proteins in absence of *uku70* or *uku80* was analyzed. For this, all the strains were grown on $MMNO_3$ and afterwards were grown overnight under non-*uku*-expressing conditions. As it can be seen in figure 57 and 58, no Rad51 foci were observed in the wild type strain and therefore no co-localization with Pot1. When *uku70* or *uku80* were absent around a 70% of the Rad51 foci were located at telomeres, which indicated that there was indeed a DNA damage signaling activation at telomeres. But strikingly this Rad51 localization at telomeres dropped down to a 20% when *uku70* or *uku80* absence was accompanied by *atr1* or *chk1* deletion.

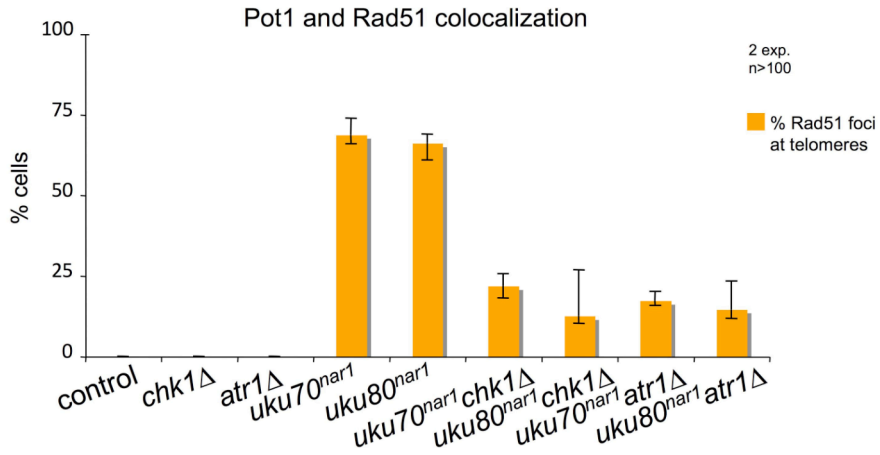


Figure 57: Quantification of Pot1 and Rad51 colocalization. Bars show the percentage of cells where Rad51 foci were located at telomeres. Two independent experiments were done and more than 100 cells were counted in each one.

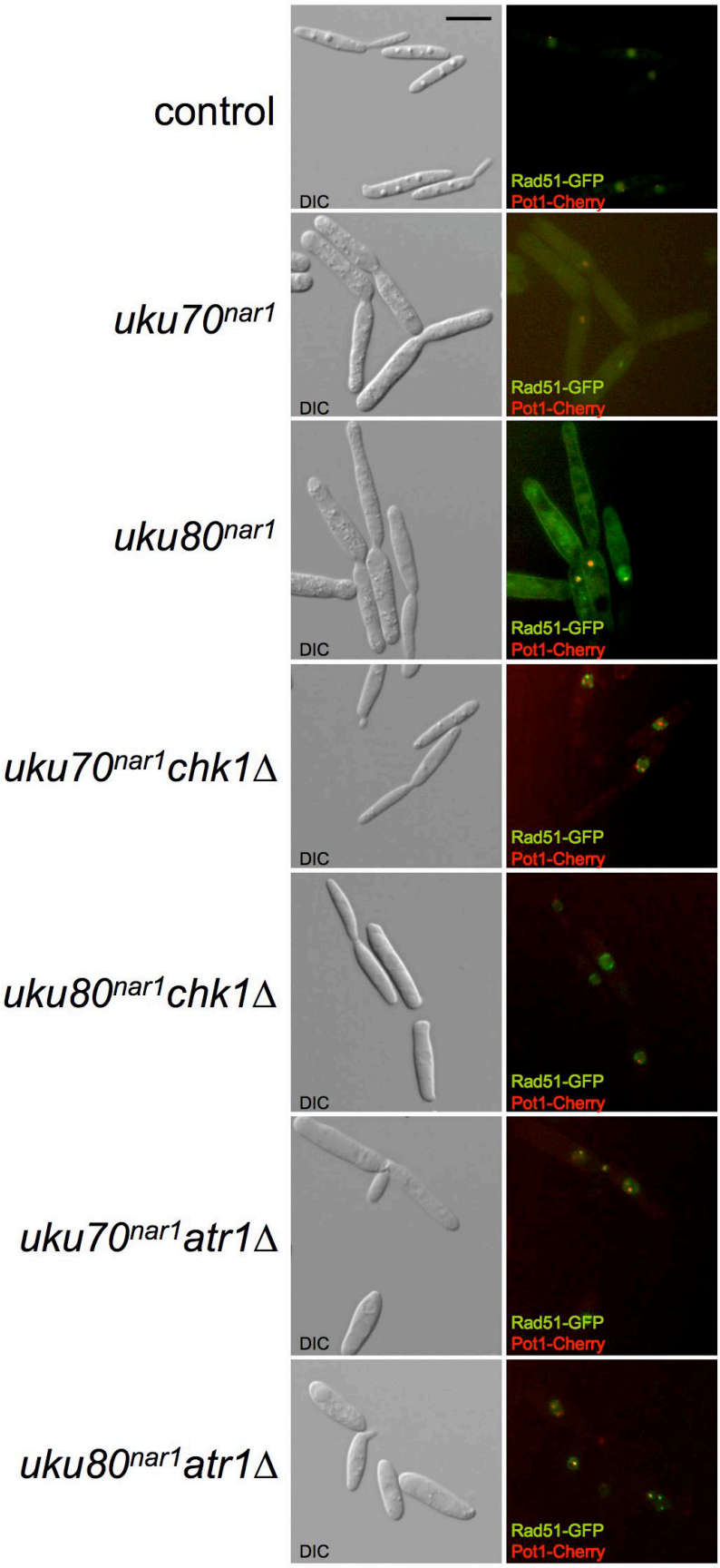


Figure 58: Rad51 and Pot1 colocalization. In absence of *uku70* or *uku80* Rad51 localizes at telomeres, while when this absence is combined with *atr1* or *chk1* deletion Rad51 location at telomeres diminish. Cells were grown on YPD overnight. Bar = 10μm

This decrease in Rad51 localization at telomeres showed that this localization was not fully dependent on the Atr1-Chk1 axis from the DNA damage response pathway.

5. Uku70/80 depletion activates homologous recombination at telomeres

Rad51 nuclear foci formation used as a DNA damage reporter also marks the repair factories in the nucleus due to its role in the homologous recombination repair pathway (Meister et al., 2003). In plants and mammals, telomeres have been shown to fold its single-stranded 3' G overhang into a structure called t-loop which parallels the first steps of homologous recombination (Cesare et al., 2003; Griffith et al., 1999). T-loops are formed in the telomeric repeats region that in most eukaryotes consists of a short tandemly repeated G-rich sequence. This structure is protected against homologous recombination because unscheduled resolution of a t-loop results in a circular telomeric molecule or t-circle (Wang et al., 2004). In mammals, plants and *C. albicans*, Ku deletion resulted in formation of telomeric circles (Chico et al., 2011; Wang et al., 2009; Zellinger et al., 2007). In *U. maydis* the telomere repeat is TTAGGG and is found tandemly repeated at least 37 times at the chromosome termini (Guzman and Sanchez, 1994). Immediately adjacent to the telomeric repeats an Ustilago Telomere-Associated Sequence or UTAS is found in all *U. maydis* chromosomes. Therefore we wondered if absence of *uku70* and *uku80* was related to the activation of homologous recombination at the chromosomal ends. For this, we analyzed t-circles formation by 2D gels in wild type and $uku70^{nar1}$ cells. By this procedure t-circles can be observed as an arch over the main migration arch. After running the gel, the membrane was hybridized with a $[TTAGGG]_5$ oligo. In the control cells, three different telomere sizes could be observed and no circular DNA was seen. Contrary to what could be observed in the absence of *uku70*, where the presence of t-circles clearly appeared. Subsequently we wonder whether the reduction in the Rad51 localization at telomeres upon *atr1* or *chk1* deletion was coupled to a homologous recombination decline at telomeres. Therefore $uku70^{nar1}atr1\Delta$ and $uku70^{nar1}chk1\Delta$ cells were analyzed for t-circle formation by 2D gels (Fig. 59). Accordingly to the Rad51 data, *chk1* and *atr1* deletion seemed to lead to a less t-circle formation when *uku70* was down-regulated.

Our results had showed a correlation between Rad51 levels and t-circles formation. Rad51 is loaded into single-stranded DNA by Brh2, as an initial step in the homologous recombination process (Yang et al., 2005) and therefore we wondered if Brh2 was involved in the formation of t-circles. For this, *uku70^{nar1}brh2Δ* and *uku70^{nar1}chk1Δbrh2Δ* strains were checked on 2D gels. Interestingly in the *uku70^{nar1}brh2Δ* cells, t-circles could be also observed, which pointed out that the homologous recombination activation at telomeres was Brh2 independent.

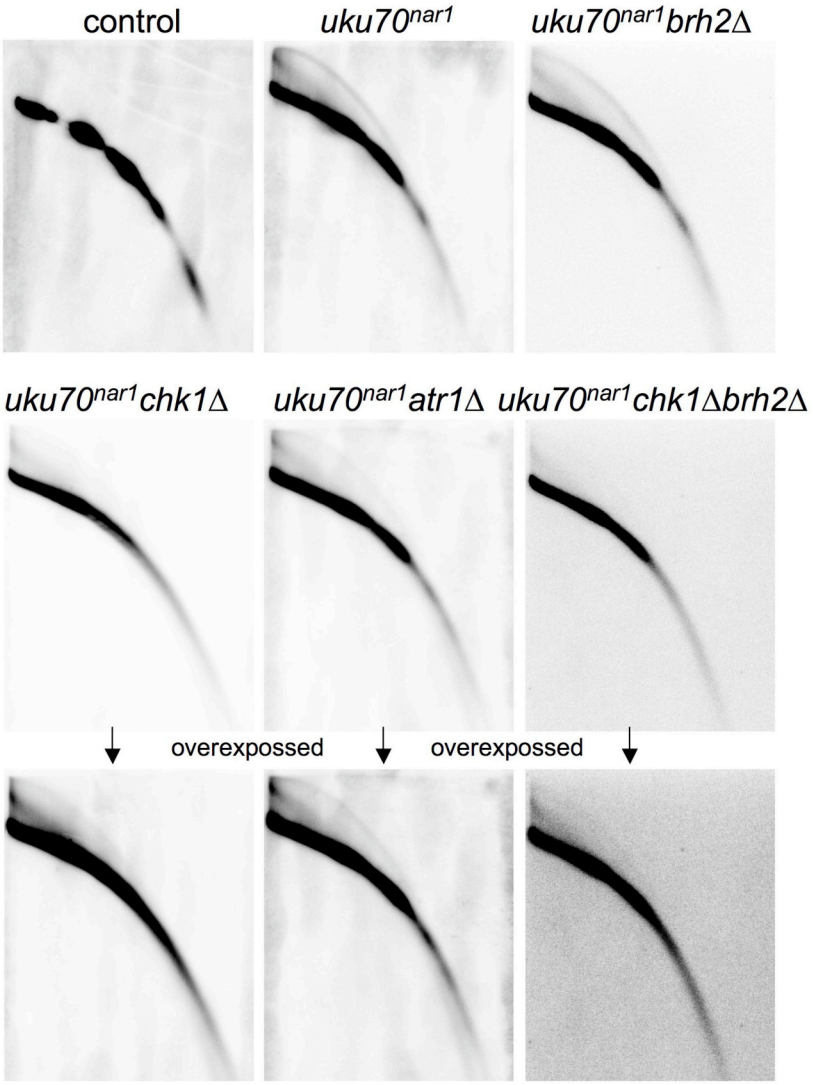
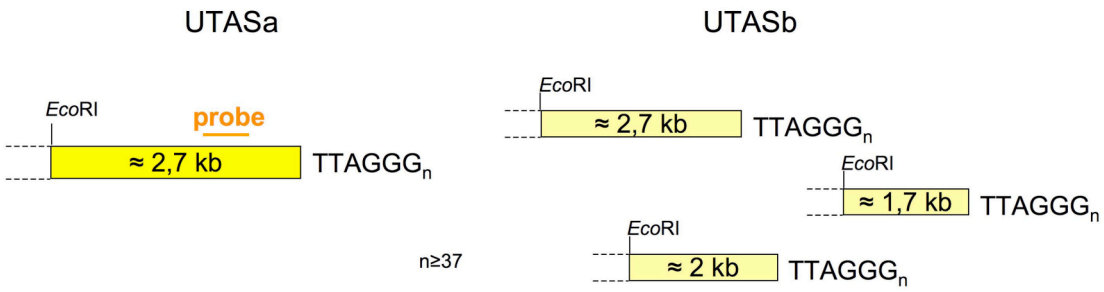


Figure 59: *uku70* absence correlates with t-circle formation. 2D gels from the strains mentioned above where the presence of t-circles can be observed.

Two pathways involved in telomere maintenance have been described: one carried out by the reverse transcriptase telomerase and the other one is a recombination-dependent mechanism known as Alternative Telomere Lengthening (ALT) (Lundblad, 2002). In yeast and mammals this homologous recombination process is the responsible

for Telomere Rapid Deletion (TRD) that leads to shorter telomeres (Bucholc et al., 2001), while in *C. albicans* and *A. thaliana*, the activation of this pathway resulted in longer telomeres (Chico et al., 2011; Zellinger et al., 2007). So cells that show an elevated level of t-circles could indicate that either ALT or TRD is occurring. To elucidate which of these processes where taking place in *U. maydis*, telomere size was analyzed by a 1D electrophoresis gel. In *U. maydis*, as in *S. cerevisiae*, two different UTAS sequences have been described to appear at the telomeres, UTASa and UTASb, although there is no clue about their distribution along the 23 chromosomes (Sanchez-Alonso and Guzman, 1998). The DNA transferred to the membrane from the gel was hybridized with a 500bp probe that identified the UTASa (Fig. 60A). As it is shown in figure 60B, *uku70* absence was related to the appearance of a smear above the 4kb marker, which was associated with slightly longer telomere fragments. This smear could not be detected in any of the single deleted mutants used as control. In the *uku70^{nar1}chk1Δ* the smear was bigger than in the rest of the double or triple mutants.

A



B

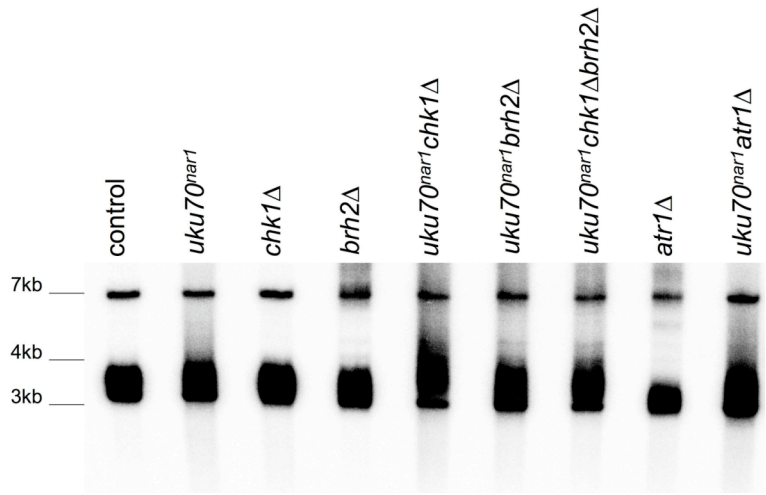


Figure 60: Ku absence in *U. maydis* leads to a telomere lengthening. A) Scheme showing the different types of UTAS present in *U. maydis* as well as the specificity of the used probe. B) Southern blot where the lengthening of the telomeres in the absence of *uku70* can be observed.

In all the strains a conserved band of 7kb was present, this could be due to the non-exclusive location of the UTASa at telomeres.

6. *uku70/80* essentiality suppressors

As we have just seen *uku70* and *uku80* essentiality could be suppressed by the deletion of *chk1* or *atr1*. We wondered if there would be more genes from the DNA damage checkpoint pathway that could suppress this essentiality. Previously, when we characterized part the DNA damage checkpoint pathway in *U. maydis,* a two-hybrid assay using *chk1* and *atr1* as baits against a *U. maydis* genomic DNA library was done with no results. It has been described that *in vivo* Ku forms an extremely stable heterodimeric Ku70/Ku80 complex and that Ku70 deficient cells have very low levels of Ku80 and vice versa (Gu et al., 1997). Therefore a screening to find other *uku70* and *uku80* essentiality suppressors was planned by using the $uku70^{nar1}$ strain. Firstly, $uku70^{nar1}$ cells were grown on *uku70* expressing conditions overnight, to be subsequently grown on rich media for 6 hours to allow a decrease in the *uku70* expression levels within the cell and avoid the formation of background in the following steps that would prevent us from picking the good colonies. After this, the culture was split on YPD plates and these were subjected to a random mutagenesis with UV radiation. From these plates 520 single colonies were isolated and streaked on YPD and YPD with HU. Only 20 colonies that could grow on YPD but failed to do so on YPD + HU were selected. These 20 colonies were crossed with *chk1Δ* and *atr1Δ* compatible strains on CMD + charcoal. From these crosses diploids were selectively isolated by their antibiotic resistance. Subsequently, the diploids were checked for complementation by their ability to grow in presence of HU. Those diploids that could not grow on HU showed that the mutagenesis had affected their *chk1* or *atr1* genes respectively (see Table 6).

Gene complementation	Number of colonies
atr1	3
chk1	3
Unknown	13

Table 6: Screening results showing the number of colonies whose mutation we were able to identify by complementation analysis.

Those colonies whose mutation could not be elucidated by a cross with a compatible strain were transformed with an *U. maydis* genomic DNA library. The identification of these genes is still ongoing.

Discussion

In this work we have characterized three different processes in which the Atr1-Chk1 regulation axis is involved. Two of them are related to DNA damage responses while the other seems to be independent from the presence of damaged DNA. A discussion of the most remarkable aspects of this study comes below.

DNA damage response pathway in *U. maydis*

In eukaryotes several pathways are involved in the DNA damage response. In *U. maydis* the knowledge about these pathways is very scarce. There is only one chekpoint kinase, homologue to Chk1 in which all the DNA damage signals would converse and upon its activation, Cdc25 becomes phosphorylated (Perez-Martin, 2009). This phosphorylation of Cdc25 produces a cell cycle arrest. When characterizing the DNA damage response pathways in *U. maydis*, only homologues for the two main kinases that recognized the different types of damage, ATM and ATR could be found. None of the mediators that participate in the transmission of the signal to Chk1, like Claspin, 53BP1…could be identified through the homology search. It is known that these mediators are not well conserved through sequence homology (Melo and Toczyski, 2002), so this could be the reason why we couldn't find them rather than their absence in *U. maydis* genome. Another important member of this pathway, essential in ATR activation, which is ATRIP, is also not preserved by sequence homology (Cortez et al., 2001) and therefore could not be found. To try to identify some of these members a two-hybrid assay was done using Chk1 and the N-terminal region of Atr1 (ATRIP interacting domain) as baits against a *U. maydis* genomic DNA library. From this assay none of the proteins that we were looking for were found. Therefore we continued characterizing the DNA damage response pathway through the members that we had already identified.

We have shown that Atm1 is essential in *U. maydis*, a striking peculiarity, since until now no Atm1 essentiality had been described in any organism. Only *Drosophila melanogaster* ATM deficient flies died as pupae or eclosed with eye and wing abnormalities. In this case, cells exhibited increased spontaneous chromosomal telomere fusions and p53-dependent apoptosis (Song et al., 2004). ATM and ATR essentiality is very variable among model organisms. There are systems where neither of them is essential as in *S. pombe*, *Aspergillus nidulans*, *Neurospora crassa* (Bentley et al., 1996; Hofmann and Harris, 2000; Malavazi et al., 2006; Naito et al., 1998; Wakabayashi et

al., 2010). And other model organisms where ATR is the essential gene like in *S. cerevisia*e and mouse (de Klein et al., 2000; Zheng et al., 1993). To assess Atm1 activity in *U. maydis* two different approaches were designed. First, a construct to exchange its endogenous promoter for the inducible promoter P_{nar1} was done. And second, two residues from the kinase domain were substituted by alanine (D2942 and N2947) in order to create a kinase-dead allele, which subsequently was placed under the inducible P_{crg1} to achieve a dominant negative allele. None of these approaches worked out. So no clues about Atm1 role in *U. maydis* were found. Only a possible role in DNA damage response could be inferred through *atr1* deletion. In *atr1Δ* cells treated with phleomycin, Chk1 was activated and this activation was followed by its location at the nucleus and the presence of the phosphorylated protein form. Phelomycin treatment provokes single and double strand breaks formation in the cell (Steighner and Povirk, 1990) and this can be sensed by ATM in other organisms. Also a possible role of Atm1 in controlling cell cycle progression could explain the *atr1Δ* phenotype, since the presence of long filamentous cells was reduced upon *chk1* deletion. Given that *atr1Δ* cells are impaired in DNA damage recognition, these cells would accumulate endogenous damage until a certain damage threshold was reached and being unable to repair it, the cells would undergo a Chk1 dependent cell cycle arrest.

Atr1 role in *U. maydis* was more deeply studied. As seen in the results, it is not essential and its deletion phenotype resembles very much to those previously described in other model organisms. We could also observe that Atr1 is the only kinase that activates Chk1 in response to the replication stress induced upon HU treatment in *U. maydis*, which couples with Atr1 described role. Atr1 deletion also provoked sensitivity to all DNA damage agents tested in the cells. These agents included UV-radiation, γ-radiation and MMS, and induced different kinds of DNA insults. Atr1 role in sensing these damages can be direct or indirect. A direct role would be in the case that single-stranded DNA was produced by either the genotoxic treatment or as a defect derived from the treatment, which in any case would lead to RPA accumulation, critical for ATR activation. An indirect activation of ATR would occur as a result of the activation of the DNA repair response triggered by ATM. Activation of the DNA repair mechanisms can produce structures that activate ATR (Ciccia and Elledge, 2010). As a concluding remark for the DNA damage response pathway in *U. maydis* we propose the following model shown in figure 61.

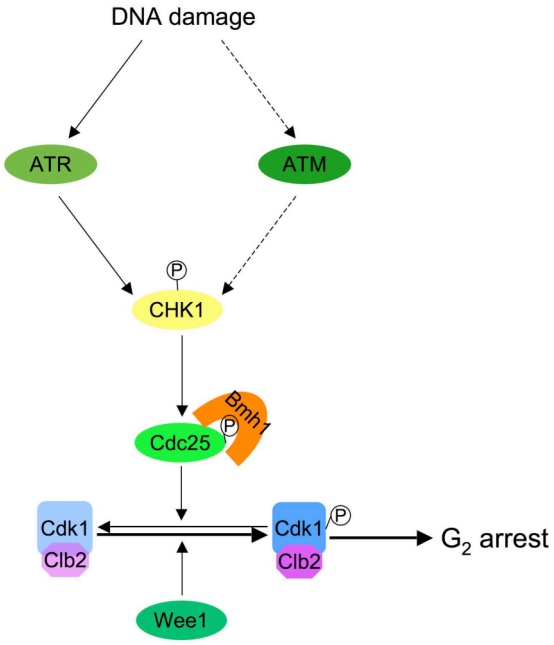


Figure 61: Model for DNA damage response in *U. maydis*.

Role of the Atr1-Chk1 axis during dikaryon formation

U. maydis is a dimorphic fungus where all the changes that are associated with its sexual development and its virulence are tightly controlled by the cell cycle. The stimuli sensed by the cells that lead to a change in its way of growth are induced by the environmental conditions followed by the expression of the mating genes. From the two mating loci that are present in *U. maydis* and determine its compatibility this work has focused in the *b* locus that codes for a transcriptional factor. The formation of this b-homeodomain protein triggers a series of changes in the cell as filamentous growth and a cell cycle arrest in G_2 phase. This cell cycle arrest has previously been shown that depends on the activation of a DNA damage checkpoint kinase, Chk1 that inhibits the entry into mitosis by an inhibitory phosphorylation of Cdc25 (Mielnichuk et al., 2009). In this work we showed that Chk1 phosphorylation induced by the b-complex is dependent on Atr1 and occurs in the same residues that are phosphorylated upon a DNA damage response. It is worth noting that no massive DNA damage could be observed: Rad51 foci formation in presence of the b-complex was used as a reporter of cellular DNA repair factories and no foci were observed. However, the absence of these foci does not discard the absence of DNA damage as the source of the Atr1-Chk1 axis activation. Several repair mechanisms are present in the cell apart from HR, for which Rad51 presence is essential. These mechanisms are the base excision repair or BER, the nucleotide excision repair or NER and the mismatch repair or MMR. Since in all these

pathways RPA is involved, and single stranded DNA coated with RPA activate ATR, these repair mechanisms could also lead to the activation of the Atr1-Chk1 axis (Zou et al., 2006). So it could be possible that the presence of damage induced by gene products regulated by b heterodimer, was responsible of the DNA damage cascade during the induction of the virulence program in *U. maydis*. Recently several reports show a link between the DNA damage response and the cell differentiation process. Examples of these processes are the programmed induction and subsequent repair of DSBs during B cell differentiation or the activation of the DNA damage response induced by DSBs in neuronal differentiation (Sherman et al., 2011).

On the other hand reports have been published showing the activation of the Atr1-Chk1 pathway as a response to developmental signals during embyogenesis in *Drosophila melanogaster* (Sibon et al., 1997) and in *Caenorhabditis elegans* (Brauchle et al., 2003). Interestingly, these works propose the idea that the damage independent activation of the Atr1-Chk1 pathway occurs in order to regulate the timing of cell divisions. This idea leads us to think that maybe something similar could be occurring during dikaryon cell division. We have seen that either in *U. maydis* or in *C. cinerea*, Atr1 and Chk1 clearly affected the dikaryon cell cycle. In both organisms the presence of clamps and nuclei per cell was impaired upon either Atr1 or Chk1 depletion. So it seems that in their absence aberrant mitosis occur. Dikaryon cell division involves development of clamp cells, synchronized nuclear division and sorting of the divided nuclei to ensure that each daughter cell inherits a balance of each parental genome (Casselton and Zolan, 2002). In *U. maydis*, b-complex is not only required for the establishment of the infective filament during the initial steps of the pathogenic stage but it is also required for synchronization between cell cycle and cytokinesis during *in planta* proliferation (Wahl et al., 2010). This role seems to be conserved among tetrapolar basidiomycetes that spend part of their life cycle as dikaryons since in *C. cinerea* A proteins regulate the conjugate nuclear division and the clamp formation. Cells with equal A proteins in *C. cinerea* present variable number of nuclei, from 0 to more than 2, and no clamps, something similar to the effect observed in *U. maydis* with common b proteins (Casselton, 1978). In *C. cinerea* clamp formation starts with the early phases of mitosis and at the same time that the clamp forms, the nuclei start to locate in parallel (Tanabe and Kamada, 1994). Once mitosis has finished the nuclei start to move along the cell keeping a constant distance between them until a certain moment when they stop (Tanabe and Kamada, 1996). During this stop nuclei start to get closer

and to prepare for the mitosis, while the cell tip continues growing. It is not known when DNA replication takes place but we propose a model where this stop followed by the bringing together of the nuclei would occur in G_2 phase. The positioning of the nucleus previously to mitosis in the clamp cell would be reminiscent of the movement of the diploid nucleus into the bud during vegetative cell division in *U. maydis*, process that takes place at the G_2/M transition (Straube et al., 2005).

In *U. maydis* we have seen that an impaired dikaryon cell cycle leads to a defect in proliferation inside the plant, and most likely as a consequence, small tumors and no spore formation could be observed. Similarly, in *C. cinerea*, a correct dikaryon formation is essential for fruiting-body development. Several mutants with defects in the different steps of fruiting body development have been described, although neither of these mutants seemed to have defects in dikaryon cell cycle (Kues, 2000). Except for the *smc1* gene, a cohesin subunit of the structural maintenance of chromosomes (SMC) protein family, which has recently been characterized in *C. cinerea*. Cohesins hold sister chromatids together until the anaphase when cleavage of the complex enables their separation. In. *C. cinerea* apart from a defect in the dikaryon mitosis it has been shown that these mutants were delayed in the hyphal knot development and these never developed into primordia (Muraguchi et al., 2008).

Since Atr1 and Chk1 seem to supply the cell with time enough to allow the "nuclear ballet" some questions arise, How does the homeodomain proteins control the Atr1-Chk1 pathway? And how is the Atr1-Chk1 pathway cyclic activation/deactivation coupled to dikaryon cell cycle transitions? The easiest answer to the first question would be that as these homeoproteins are transcription factors, they could directly regulate *chk1* or *atr1* transcription. At least in *U. maydis* it had previously been seen that this was not the case, since upon b-induction no upregulation of neither of these genes could be observed (Heimel et al., 2010b). Another option would be the presence of DNA damage as the cause of the Atr1-Chk1 pathway activation. But this seems not to be either the case, since DNA self-harm every cell cycle doesn't seem to be a very smart way of survival in the cell. And also we have seen that dikaryotic growth of cells lacking Brh2, which is essential for DNA repair by homologous recombination, behaves as wild type cells, although as discussed before the presence of small DNA damage cannot be disregarded. Strikingly, one possibility could be that something similar to what two recent reports have shown might be occurring during dikaryon cell cycle. These reports showed that activation of the DNA damage response cascade could be

triggered in absence of DNA damage by stable association of elements of the cascade with the chromatin (Bonilla et al., 2008; Soutoglou and Misteli, 2008).

In the shut off of this signal cascade two different levels of regulation would be needed: regulation of the transcriptional activity of the b heterodimer and the absence of the Ar1-Chk1 signaling. Downregulation of b heterodimer could occur through the activity of the Clp1 (or Clampless1) protein. This protein was firstly described in *C. cinerea* as essential for A-regulated sexual development since its expression could only be detected when the homeodomain factors were present and its deletion led to an absence in clamp and fruiting body formation probably associated with an aberrant distribution of nuclei (Inada et al., 2001). Clp1 has recently been characterized in *U. maydis* and it has been shown that its interaction with one of the subunits of the b proteins blocks b-dependent functions as the b-dependent G_2 cell cycle arrest (Heimel et al., 2010a). Interestingly, *clp1* transcription is also indirectly controlled by the b heterodimer, via the induction of the transcriptional activator Rbf1 in *U. maydis*. The second regulation level in charge of Atr1-Chk1 downregulation is unknown. A possibility could be the role of the PTEN/AKT pathway. In this pathway described in mammals, AKT overrides DNA damage-induced G_2 arrest via an inhibitory phosphorylation of Chk1 and in absence of PTEN Chk1 cytoplasmic sequestration is increased (Puc et al., 2005; Shtivelman et al., 2002). Homologues of these two proteins in *U.maydis* are b-dependent upregulated. Even more, *ukb1*, the putative ortholog of PKB in *U. maydis*, is needed for *in planta* proliferation since its depletion produces small tumor formation and absence of teliospores, a phenotype that resembles very much to the one observed upon either *chk1* or *atr1* deletion (Abramovitch et al., 2002).

Taking all this ideas into account we propose a model in which activation and deactivation cycles of the Atr1-Chk1 axis would control the dikaryotic cell cycle. Although the activation of the Atr1-Chk1 pathway is not clear, we have proposed two mechanisms that could controlled the shut off of the pathway, both of them dependent on the transcriptional activator Rbf1. This model appears in figure 62.

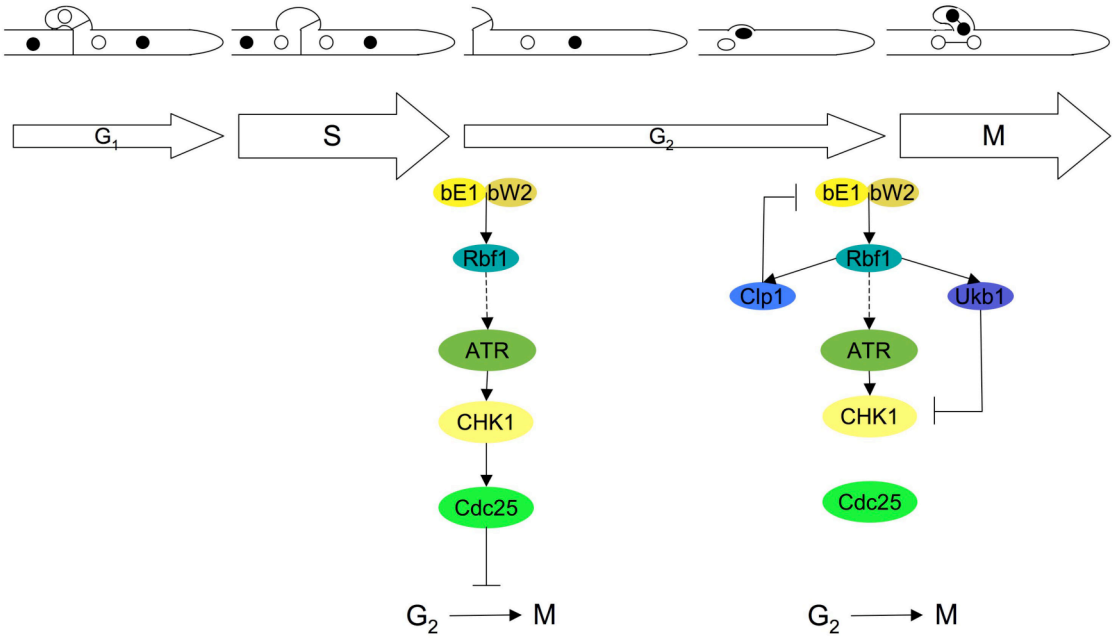


Figure 62: Dikaryon cell cycle regulation dependent on the Atr1-Chk1 axis in Basidiomycetes

Telomere damage signaling through the Atr1-Chk1 axis

Ku proteins have been studied in fungi for a long time as a way to increase HR. Since its main described role for a long time was in NHEJ, their deletion was shown to be a good way for increasing gene targeting by HR (Nakazawa et al., 2011; Nayak et al., 2006). Previous attempts in characterizing these genes had suggested a possible essentiality in *U. maydis* (W.K. Holloman personal communication). Since *ligase IV* mutants are viable, essentiality could be related to its role in telomeres. In this work we have shown that Uku70 and Uku80 essentiality is related to the activation of a checkpoint cell cycle arrest dependent on the Atr1-Chk1 pathway. Even more, cell viability is fully recovered upon *chk1* deletion in absence of the Uku proteins, and cells show a normal appearance. A certain viability recovery could also be observed upon *atr1* deletion, although this was not as clear as the one observed with *chk1*, probably due to the presence of Atm1, which can still act as a Chk1 activator in the cell cycle arrest. Interestingly, Ku essentiality has only been reported in humans but clues about this essentiality are unknown for the moment (Wang et al., 2009).

Why Uku heterodimer absence activates a checkpoint response that leads to a cell cycle arrest? The easiest answer would be related to the telomere-protecting role that has been designated for Ku proteins. In absence of Ku proteins the telomeres are

deprotected and are susceptible to exonuclease attack and this damage induces the DNA damage response. In *S. cerevisiae* Yku70 or Yku80 deletion is viable when the cells are grown at permissive temperatures although cells present short telomeres, ssDNA at the telomeric repeats, decreased telomeric silencing and altered telomere localization. When cells are grown at 37ºC they are not able to form colonies due to an activation of the checkpoint pathway in response to the accumulation of ssDNA at the telomeres. This checkpoint activation is triggered by Atr1, Chk1, and Rad9 and leads to a cell cycle arrest. This arrest can be partially suppressed by the overexpression of telomerase subunits or by the deletion of Exo1, an exonuclease that generates ssDNA at telomeres (Maringele and Lydall, 2002). A similar activity for Exo1 in telomere degradation has been observed in *S. pombe* (Tomita et al., 2003). From previous work in trying to characterize Ku proteins in *U. maydis* we knew that *exo1* deletion could not overrode Uku essentiality (W.K. Holloman personal communication). So either degradation was being carried out by other exonuclease like Mre11 or this was not the type of damage that was triggering the checkpoint dependent cell cycle arrest. We believe that this second option is the one that is occurring in *U. maydis* since upon Uku70 absence no telomere shortening was observed. In summary, these observations show that Uku70/80 role in telomeres in U. maydis seems to be very different from other organisms and mainly from *S. cerevisiae*.

Another possibility would be related to the telomere structure. There is no clue about the proteins that are involved in the telosome or shelterin formation in *U. maydis*, although some of the proteins that are present in other organisms have been identified by sequence homology and a role for them has been inferred (Sanchez-Alonso and Guzman, 2008). Accordingly to these analyses, *U. maydis* telosome would resemble very much to *S. pombe*. In order to maintain genomic integrity, telomeres must be able to prevent DNA repair and DNA damage checkpoint proteins from causing unwanted effects or from eliciting cell cycle arrest, and at the same time telomeres must provide access to telomerase to prevent loss of telomeric DNA (Blackburn, 2001). In eukaryotes it is known that ATM and ATR are required for maintenance of stable telomeres, although the molecular basis has still to be elucidated. In *S. pombe* ATM and ATR homologues are required for efficient recruitment of the shelterin components, which in turn promotes recruitment of telomerase, and for a correct protection of the telomeres against recombination (Moser et al., 2009). So how come ATR and ATM presence at the telomeres does not elicit a damage checkpoint? In *S. pombe* it has been shown that

telomeres instead of preventing the detection of the DNA damage, a chromatin-privileged region is constituted that blocks the transduction of an active checkpoint signal to Chk1 (Carneiro et al., 2010). So it could be possible that if chromatin organization at telomeres in *U. maydis* showed similarity to *S. pombe*'s, upon Uku absence, the alteration in the chromatin conformation could facilitate the transmission of the checkpoint signal to Chk1 triggering a cell cycle arrest in absence of DNA damage.

Besides the heterogenicity of Ku studied roles in all the model organisms, a common feature is share among them. From budding yeast to mammals Ku has been shown to inhibit Rad51 recruitment to the telomeres, in order to inhibit homologous recombination (Celli et al., 2006; Kibe et al., 2003; Polotnianka et al., 1998). As a eukaryote common feature, this role is also conserved in *U. maydis*, since upon Uku absence we have observed t-circle formation in the telomeres. A strikingly observation was that this homologous recombination pathway seemed to be not dependent on Brh2. So, who is triggering homologous recombination at telomeres in *U. maydis*? Homology-directed repair is done by proteins from the RAD52 family, from yeast to humans it has been shown that these proteins and the Rad51 paralogues can facilitate loading of Rad51 onto single-stranded DNA coated with RPA to form the nucleoprotein filament that constitutes the active homology search engine and DNA strand exchange machine. In *U. maydis* a BRCA2 homologue, Brh2, and a RAD52 orthologue, Rad52, are present together with a Rad51 paralogue, Rec2. It seems that loading of Rad51 on RPA coated single-stranded DNA is mainly done by Brh2 rather than Rad52. Although it has also been observed that Rec2 and Rad52 promote and maintain Rad51 filament stability (Kojic et al., 2002; Kojic et al., 2008; Kojic et al., 2006). So both of these proteins could be good targets to analyze their role in this homologous recombination process at telomeres.

Another interesting observation was the reduction of t-circle formation and Rad51 localization at telomeres in absence of either Atr1 or Chk1. With the results obtained it seemed that the presence of Atr1 and Chk1 in absence of Uku heterodimer promoted t-circle formation at telomeres. We have no explanation for this effect. In mammals deficiency in the shelterin complex formation activates the Atr1-Chk1 checkpoint pathway accompanied of the NHEJ pathway activation. But when this deficient shelterin complex is coupled with either ATM or ATR deletion a reduction in telomere fusions is observed (Denchi and de Lange, 2007). Something similar could be

happening in *U. maydis*, but since Uku is needed for NHEJ, the effects could be observed in the HR pathway that is activated upon Uku absence.

Finally, the striking effect on telomere size in absence of Uku heterodimer deserves a discussion. A possibility that could induce such an increase in the telomere size and that could be working together with the induced homologous recombination process could be a misregulation of the telomerase. But how could this telomerase misregulation occur? In fission yeast, ATM and ATR homologues are required for telomerase recruitment. Due to the similarity of the shelterin complex between *S. pombe* and humans, regulation of the telomerase activity has been proposed to be controlled by these proteins (Bianchi and Shore, 2008). Taking this into account it could be that Uku absence altered the shelterin structure and this in turn would lead to a misregulation of the telomerase producing elongated telomeres. The increase would be bigger in those cells where Uku absence was coupled to *chk1* deletion, since Atm1 and Atr1 are both present to recruit the telomerase, while it would be smaller in absence of Atr1, since only Atm1 would recruit telomerase.

Another possibility would be that the lengthening relied only in homologous recombination. Homologous recombination at telomeres has been proposed as an alternative mechanism to maintain telomere length in cells where telomerase is not active (Lundblad, 2002). Due to the activation of the cell cycle arrest coupled with the absence of the Uku heterodimer, cells do not have enough time to show the extremely long telomeric size that has been observed in other organisms as *Candida albicans* or *Arabidopsis thaliana*, and instead a slight increased in the size can be observed. This could explain why Uku absence alone or coupled with *brh2* deletion show a similar increment in telomeric size, while when Uku absence was coupled with *atr1* or *chk1* deletion, the increment was proportional to the extent of their capability to override the essentiality. If we take together the results from the telomere size and the formation of t-circles we see that a less presence of t-circles couples with a bigger increase in telomeric size. Formation of t-circles and integration of the circles into the telomeres is a homologous recombination dependent process. So it could well be that in presence of Atr1 and Chk1 the formation of t-circles was more favorable than the integration of them on the DNA. And similarly, upon *atr1* or *chk1* deletion the balance was tipped in the integration of the t-circles.

With all these observations, we propose a model of the different processed that are regulated by the Uku heterodimer in *U. maydis* telomeres (Fig. 63).

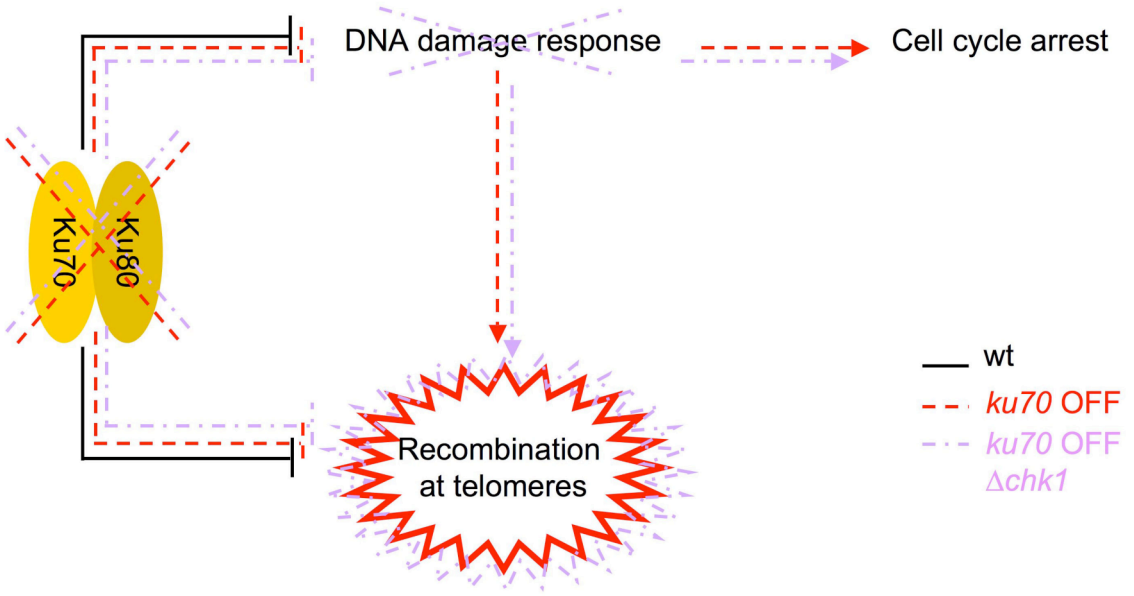


Figure 63: Different pathways regulated by the Uku heterodimer in *U. maydis*.

Conclusions

1. Atr1 in *U. maydis* is involved in activating the DNA damage response in presence of replication stress.

2. Chk1 activation in response to replication stress is fully dependent on Atr1.

3. Atm1 is an essential gene in *U. maydis*.

4. b-dependent Chk1 activation relies on the same residues that are phosphorylated in the DNA damage response and is controlled by Atr1.

5. Infective filament b-dependent cell cycle arrest is partially controlled by Atr1 and does not correlate with a massive DNA damage response.

6. Atr1 and Chk1 control the dikaryon cell cycle and defects in this process are the cause of a defective *in planta* proliferation of *U. maydis* and a defective mature fruiting body formation in *C. cinerea*.

7. Uku70 and Uku80 at telomeres inhibit Atr1-Chk1-dependent checkpoint response activation.

8. Essentiality derived from Uku70 and Uku80 absence can be fully overridden through Chk1 deletion and partially through Atr1 deletion

9. Uku70 and Uku80 inhibit homologous recombination at telomeres.

Conclusiones en Español

1. En *U. maydis*, Atr1 participa en la activación de la respuesta a daño en el ADN en presencia de estrés replicativo.

2. La activación de Chk1 en respuesta a estrés replicativo depende exclusivamente de Atr1.

3. Atm1 es un gen esencial en *U. maydis*.

4. La activación de Chk1 dependiente de las proteínas b recae en los mismos residuos que son fosforilados en la respuesta al daño en el ADN y está controlada por Atr1.

5. La parada del ciclo celular dependiente de las proteínas b que tiene lugar en el filamento infectivo está controlada parcialmente por Atr1 y no se correlaciona con una respuesta masiva al daño en el ADN.

6. Atr1 y Chk1 controlan el ciclo celular del dicarionte y los defectos en este proceso son la causa de una proliferación defectuosa *in planta* de *U. maydis* y de una formación de cuerpos fructíferos maduros defectuosa en *C. cinerea*.

7. Uku70 y Uku80 inhiben la activación de la respuesta de checkpoint dependiente de Atr1-Chk1 en los telómeros.

8. La esencialidad derivada de la ausencia de Uku70 y Uku80 se puede abolir completamente mediante la deleción de Chk1 y parcialmente con la deleción de Atr1.

9. Uku70 y Uku80 inhiben la recombinación homóloga en los telómeros.

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Appendix

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The DNA Damage Response Signaling Cascade Regulates Proliferation of the Phytopathogenic Fungus *Ustilago maydis* in Planta [W]

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In the phytopathogenic fungus *Ustilago maydis*, the dikaryotic state dominates the period of growth occurring during the infectious phase. Dikaryons are cells in which two nuclei, one from each parent cell, share a single cytoplasm for a period of time without undergoing nuclear fusion. In fungal cells, maintenance of the dikaryotic state requires an intricate cell division process that often involves the formation of a structure known as the clamp connection as well as the sorting of one of the nuclei to this structure to ensure that each daughter dikaryon inherits a balance of each parental genome. Here, we describe an atypical role of the DNA damage checkpoint kinases Chk1 and Atr1 during pathogenic growth of *U. maydis*. We found that Chk1 and Atr1 collaborate to control cell cycle arrest during the induction of the virulence program in *U. maydis* and that Chk1 and Atr1 work together to control the dikaryon formation. These findings uncover a link between a widely conserved signaling cascade and the virulence program in a phytopathogen. We propose a model in which adjustment of the cell cycle by the Atr1-Chk1 axis controls fidelity in dikaryon formation. Therefore, Chk1 and Atr1 emerge as critical cell type regulators in addition to their roles in the DNA damage response.

INTRODUCTION

The sexual life cycle starts with fusion of two different haploid cells, proceeds to the formation of a diploid, and ends with meiosis to generate four haploid cells (Chen et al., 2007). However, the way in which haploid cells are brought together and the predominance of the haploid or diploid phase varies between species. In fungal cells, mating, the process equivalent to fertilization, brings together two haploid nuclei in the same cytoplasm. In some species of fungi, this process is followed by nuclear fusion, resulting in a diploid nucleus that enters meiosis immediately (as occurs in the fission yeast *Schizosaccharomyces pombe*) or that is maintained and proliferates in the diploid state (as in budding yeast *Saccharomyces cerevisiae*). However, for a large group of fungi, mating results in a dikaryon, a cell in which the two nuclei, one from each parent cell, share a single cytoplasm for a period of time without undergoing nuclear fusion. The dikaryon stage is typical in the life cycles of many fungal species primarily in the Basidiomycota, a large group that includes mushrooms, bracket fungi, and many phytopathogenic fungi, such as the maize pathogen *Ustilago maydis*.

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Establishment and maintenance of dikaryotic growth in Basidiomycete fungi is controlled by information specified at the Mating Type (*MAT*) loci, specialized regions of fungal genomes akin to the sex chromosomes of larger eukaryotes (Lee et al., 2010). Although the specific contribution of the *MAT* locus components to dikaryon formation varies among species characterized thus far, a central element common to all of them is the activation of a specific transcriptional cascade controlled by a heterodimeric homeodomain transcription factor, with components derived from the *MAT* locus of each parent. Dikaryotic maintenance involves an intricate cell division process to ensure that each dikaryon inherits a balance of each parental genome. In many dikaryotic basidiomycetes (with some exceptions, such as various Uredinomycetes; for instance, see Ikeda et al., 2003) cell division involves the formation of a specialized structure known as the clamp connection as well as the sorting of one of the nuclei to this structure. In this way, nuclear division occurs in a synchronous and independent fashion in two distinct subcellular compartments. Once nuclear division is finished, the clamp connection is resolved to reconstitute the dikaryotic status of daughter cells (Brown and Casselton, 2001; Gladfelter and Berman, 2009). Studies with *Coprinopsis cinerea* and *Schizophyllum commune* indicated that the *MAT* genes encoding homeodomain transcription factors govern nuclear pairing as well as clamp formation (Casselton and Olesnicky, 1998; Kües, 2000); therefore, a connection between *MAT* genes and cell cycle control is predicted, although the details behind these connections are largely unknown.

In *U. maydis*, the dikaryotic state dominates the period of growth occurring during the infectious phase. This state is dependent upon the *MAT*-encoded homeoprotein called the b-complex, whose subunits, bW and bE, are provided by each mating partner (Feldbrügge et al., 2004; Brefort et al., 2009). During induction of the virulence program in *U. maydis*, an infectious dikaryotic hypha is produced on the plant surface as a result of mating of a pair of compatible haploid budding cells. The infectious hypha or filament is composed of a single dikaryotic cell that is arrested in the cell cycle at G2 phase (Mielnichuk et al., 2009), and its formation is dependent on synthesis of the b-complex. The arrest is transient, and eventually the filament manages to enter the plant tissue, where it starts to proliferate while maintaining its dikaryotic status. Since mutations that abolish this transient cell cycle arrest also impair dikaryon formation (Mielnichuk et al., 2009), it is thought that b-induced cell cycle arrest is required for dikaryon establishment in *U. maydis*. In our efforts to describe the mechanisms behind this transient cell cycle arrest, we recently reported that the widely conserved Chk1 protein kinase is required for this cell cycle arrest (Mielnichuk et al., 2009). Chk1 is better known as a key signal transducer within the DNA damage response cascade (Chen and Sanchez, 2004) in a broad range of eukaryotes, including *U. maydis* (Pérez-Martín, 2009). Here, we investigated the response of Chk1 to b-complex formation. We present findings showing that Chk1 is activated through phosphorylation by the conserved upstream activating kinase Atr1 and that the Atr1-Chk1 regulatory axis serves in maintenance of dikaryotic status in planta. Because Chk1 controls cell cycle progression, we propose that b-complex–mediated activation of the Atr1-Chk1 axis is part of the mechanism responsible of coordination during a dikaryotic cell cycle.

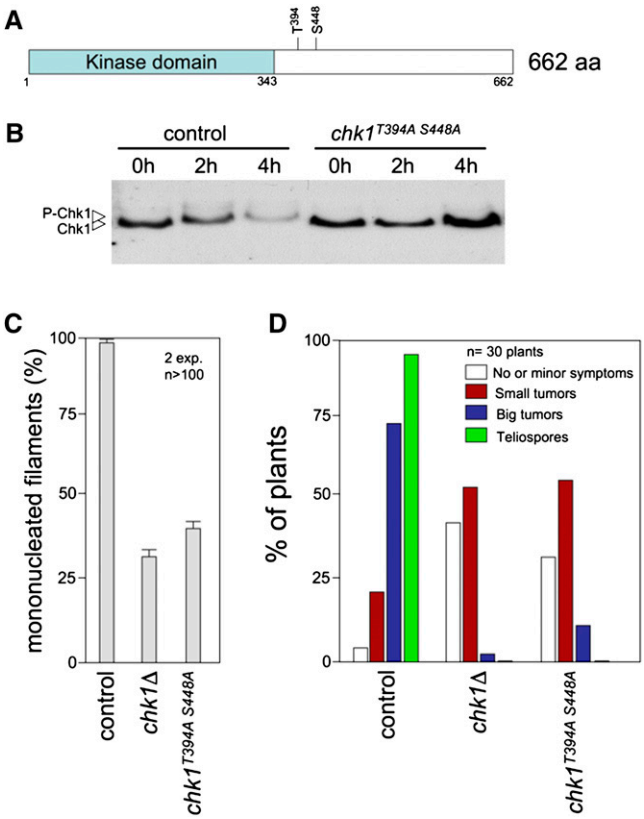

Figure 1. A *chk1* Allele Refractory to Phosphorylation Mimics the *chk1*Δ Loss-of-Function Mutation with Respect to b-Dependent Filament Cell Cycle Arrest and in Planta Proliferation.

(A) Scheme of Chk1 showing the kinase domain and two phosphorylatable residues required for Chk1 activation. aa, amino acids.

(B) In vivo phosphorylation of Chk1 during *b*-dependent filamentation. AB33-derived cells carrying an endogenous *chk1-T7* (control, UCS31) or the *chk1*$^{T394A\ S448A}$ allele (UMP183) were incubated in inducing conditions (MM-NO_3) for the indicated time (in hours). Protein extracts were immunoprecipitated with a commercial anti-T7 antibody, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-T7 antibody.

(C) AB33 (control, UCS31) and derived strains lacking the *chk1* gene (*chk1*Δ, UMP114) or carrying the *chk1*$^{T394A\ S448A}$ allele (UMP183) were incubated in inducing conditions (MM-NO_3). Cells were stained with 4′,6-diamidino-2-phenylindole to detect nuclei. The percentage of cells producing mononucleated filaments (i.e., cell cycle arrested) after 24 h of incubation is shown graphically. The graph shows the result from two independent experiments, counting more than 100 cells each. Means and SDs are shown.

(D) Quantification of symptoms in maize plants after 14 d after infection with wild-type (control, UCM350xUCM520), *chk1*Δ (UMP122xUMP129), or *chk1*$^{T394A\ S448A}$ (UMP190xUMP191) mutant crosses.

RESULTS

b-Dependent Cell Cycle Arrest Requires Chk1 Activating Phosphorylation

Assembly of the heterodimeric b-complex during dikaryon formation in *U. maydis* is concomitant with transient cell cycle arrest as well as with accumulation of phosphorylated forms of Chk1 and translocation of Chk1 into the nucleus, two hallmarks of Chk1 activation (Mielnichuk et al., 2009). Previous research (Pérez-Martín, 2009) established that in response to DNA damage, Chk1 activation results in G2 cell cycle arrest and involves phosphorylation at two residues (Thr-394 and Ser-448) located in the regulatory domain (Figure 1A). Mutant isoforms containing Ala in place of these residues could not be activated in response to DNA damage signals (Pérez-Martín, 2009). To determine whether these phosphorylation sites were also important during the cell cycle arrest associated with dikaryon formation, we measured the ability of these phosphorylation refractory Chk1 mutants to support the *b*-induced cell cycle arrest. For this, a *chk1*$^{T394A\ S448A}$ mutant allele tagged with the T7 epitope was integrated at the native locus in AB33 cells, a haploid strain that carries compatible (i.e., able to dimerize) *bE* and *bW* genes under the control of the inducible *nar1* promoter that is induced by the addition of nitrate to medium (Brachmann et al., 2001). As a control, a T7-tagged wild-type *chk1* allele was used. Upon induction, Chk1$^{T394A\ S448A}$ protein did not show the reduced electrophoretic mobility observed with wild-type Chk1 protein after induction of heterodimeric b protein (Figure 1B). Moreover, the cells carrying the nonphosphorylatable Chk1 allele were

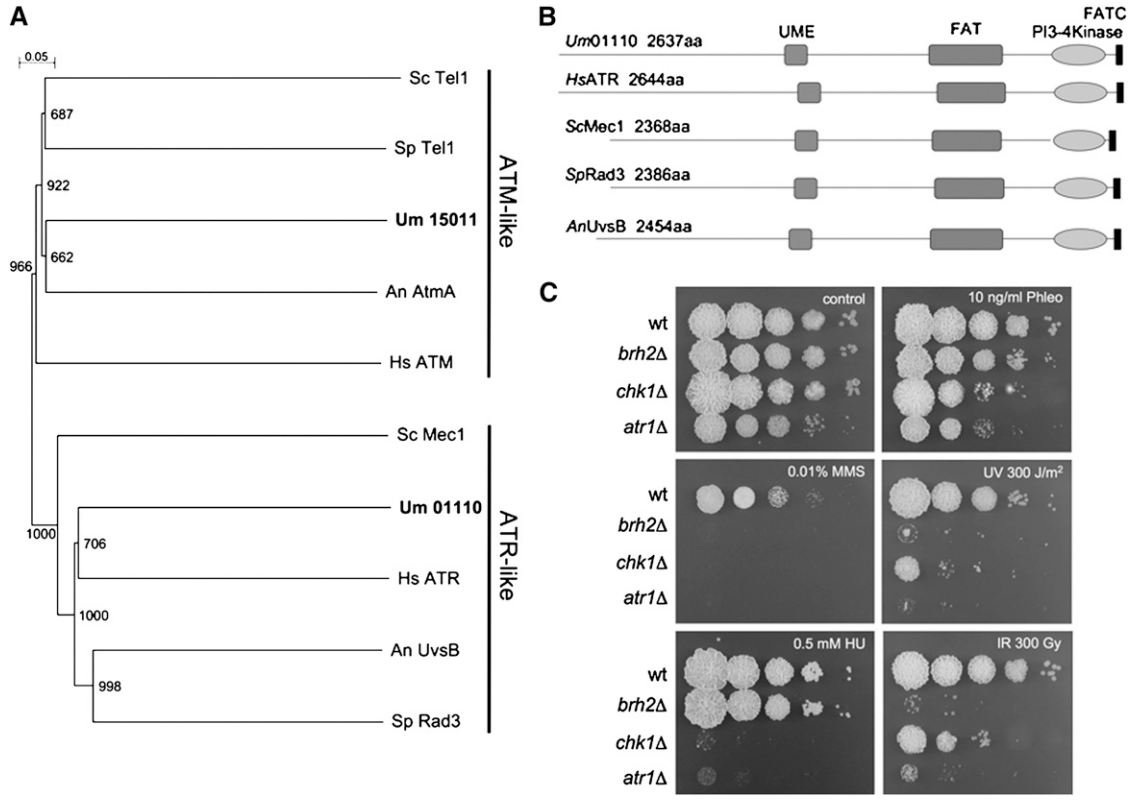

Figure 2. *U. maydis* Atr1.

(A) Dendrogram of characterized Atr1 and Atm1-like proteins. The tree was created by the distance-based minimum evolution method, based on 1000 replicates. Bootstrap values are given, and branching points and the scale bar denote substitutions per site. The proteins used were *Homo sapiens* ATR (CAA70298.1) and ATM (AAB38309.1); *S. pombe* Rad3 (SPBC216.05) and Tel1 (SPCC23B6.03c); *S. cerevisiae* Mec1 (YBR136W) and Tel1 (YBL088C); *Aspergillus nidulans* AtmA (AN0038.2) and UvsB (AN6975.2); and *U. maydis* Um15011 (Atm1) and Um01110 (Atr1).

(B) Schematic representation of the domain architecture of Atr1 proteins in different organisms. Boxes represent UME (UVSB PI-3 kinase/MEI-4/ESR1 conserved domain), FAT (FRAP/ATM/TRRAP conserved domain), the PI3-kinase domain, and FATC (FRAP/ATM/TOR C-terminal region), all of them conserved domains in ATR-like kinases.

(C) Sensitivity of cells lacking *atr1* gene (UCS9) in comparison to wild-type (wt; UCM350), *chk1*Δ (UMP122), and *brh2*Δ (UCM565) cells to different chemicals as well as IR and UV irradiation. A 10^8 cells/mL cell suspension and a series of 10-fold dilutions were spotted (2 μL per spot) onto agar plates containing the indicated drugs (HU, hydroxyurea; Phleo, phleomycin; MMS, methyl methanesulfonate). For UV ad IR sensitivity, cells were irradiated at the indicated dose after being spotted. The spots were photographed after incubation for 2 d.

impaired in cell cycle arrest just as were cells deleted for *chk1* (Figure 1C). We also analyzed the ability of compatible haploid cells carrying the *chk1*$^{T394A\ S448A}$ allele to infect plants and found that the nonphosphorylatable mutant showed similar virulence defects as those observed in cells lacking Chk1 protein (Figure 1D). In summary, these results show that the *chk1*$^{T394A\ S448A}$ mutant phenocopies all defects observed in the *chk1* null mutant, implying that phosphorylation of Chk1 at these residues is integral to the mechanism of b-dependent activation of Chk1.

Atr1 Is Required in the Response to DNA Damage in *U. maydis*

In other model systems, Chk1 phosphorylation in response to DNA damage is performed by two phosphatidylinositol 3-kinase-related kinases (PIKKs), ATM and ATR (Zhou and Elledge, 2000; Nyberg et al., 2002). Putative orthologs of these two PIKKs are present in *U. maydis* genome (Figure 2A; see Supplemental Data Set 1 online). The genes encoding Atm1 (Um15011) and Atr1 (Um01110) were identified as entries (noted in parentheses) in the manually annotated Munich Information Center for Protein Sequences *U. maydis* database (see http://mips.helmholtz-muenchen.de/genre/proj/ustilago/). To further analyze the relationships between these putative kinases and Chk1, gene deletion of the corresponding genes was attempted. Constructions containing a hygromycin resistance cassette flanked by regions located upstream and downstream of the *atr1* and *atm1* open reading frames were transformed in haploid cells. Only mutants lacking the *atr1* gene were obtained. As this result suggested that *atm1* was essential, a diploid/meiotic analysis protocol was employed. We successfully inactivated one *atm1* allele in the diploid FBD11 strain, replacing it with the hygromycin resistance cassette, generating the *atm1*Δ null allele. After sporulation, we analyzed the meiotic progeny of this strain, and we

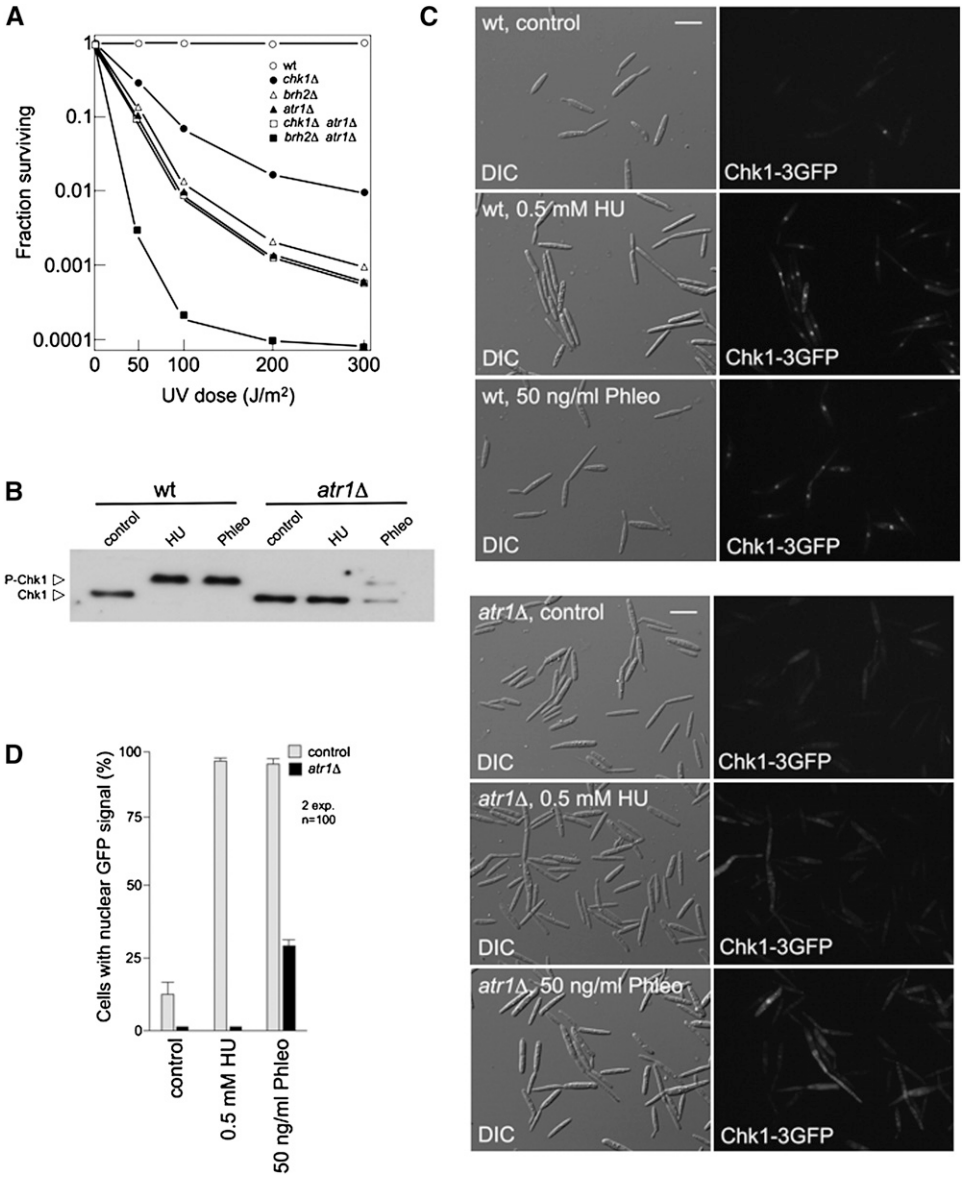

Figure 3. Atr1 Is Required for Activation of Chk1 in Response to DNA Damage in *U. maydis*.

(A) Epistasis analysis between *chk1*, *atr1*, and *brh2*. Survival curves against UV irradiation of the indicated single and double mutants were obtained. Cells were grown to late log phase, adjusted to a density of 2×10^7 cells per mL, and irradiated with UV. Survival was determined by counting colonies visible after incubation for 2 to 3 d. wt, wild type.

(B) In vivo phosphorylation of Chk1 in response to agents that induce DNA damage depends on Atr1. Wild-type (UMP162) and *atr1*Δ (UMP207) cells carrying an endogenous Chk1-T7 allele were grown with no treatment (control) or in the presence of 0.5 mM HU or 50 ng/mL phleomycin (Phleo) for 6 h. Protein extracts were immunoprecipitated with a commercial anti-T7 antibody, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-T7 antibody.

(C) Atr1 is required to localize Chk1 at the nucleus. Cell images of wild-type (UMP111) and *atr1*Δ (UCS15) strains carrying a Chk1-3GFP fusion protein after 3 h of incubation in the presence of HU or phleomycin (Phleo). DIC, differential interference contrast. Bar = 10 μm.

(D) Quantification of the cell response to DNA damage as the percentage of cells carrying a clear nuclear GFP fluorescence signal. The graph shows the result from two independent experiments, counting more than 100 cells each. Means and SDs are shown.

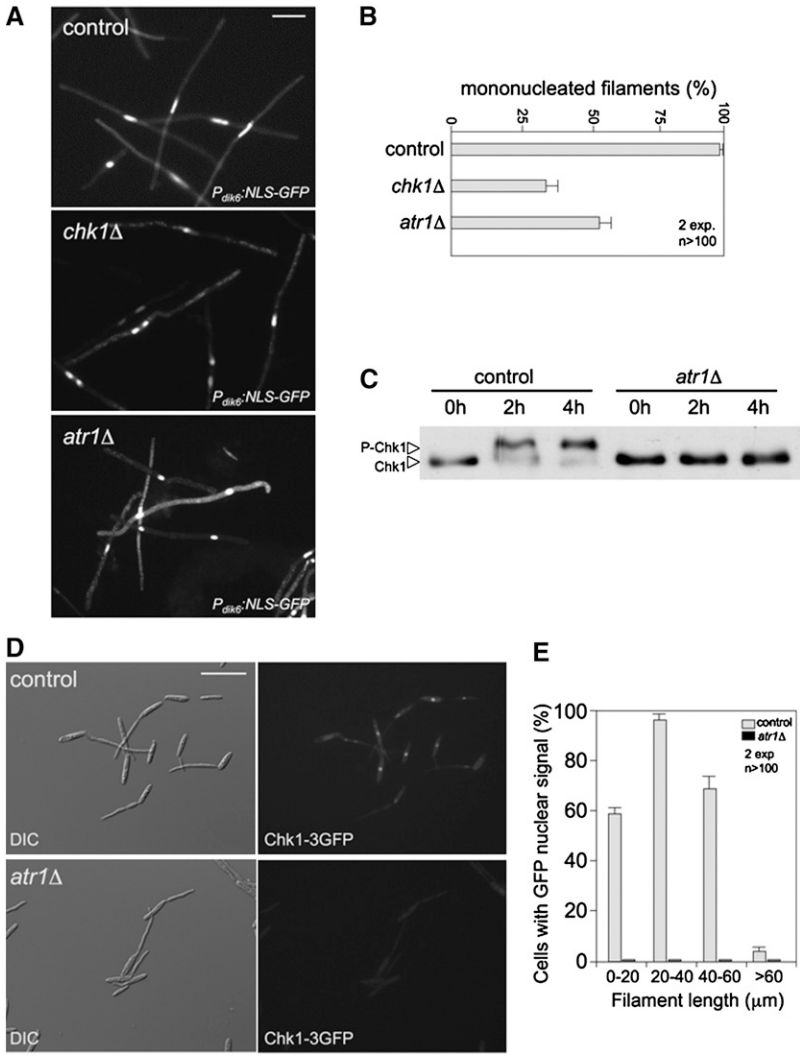

Figure 4. Atr1 Is Required for b-Dependent Cell Cycle Arrest.

(A) Cell images of control (UCS20) and a derived strain lacking the *chk1* gene (*chk1*Δ, UMP112) or *atr1* (*atr1*Δ, UCS21) incubated for 8 h in inducing conditions (MM-NO_3). Strains carried a NLS-GFP fusion under control of the *b*-dependent *dik6* promoter to detect the nucleus. Bar = 15 μm.

(B) Percentage of cells producing mononucleated filaments (i.e., cell cycle arrested) after 24 h of incubation. The graph shows the result from two independent experiments, counting more than 100 cells each. Means and SDs are shown.

(C) Dependence on *atr1* for in vivo phosphorylation of Chk1 during *b*-dependent filamentation. Control (UCS31) and *atr1*Δ (UCS32) AB33-derived cells carrying an endogenous *chk1-T7* allele were incubated in MM-NO_3 for the indicated time (in hours). Protein extracts were immunoprecipitated with a commercial anti-T7 antibody, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-T7 antibody.

(D) Atr1 is required to localize Chk1 at the nucleus in response to *b*-induction. Cell images of control (UMP133) and *atr1*Δ strain (UMP208) carrying a Chk1-3GFP fusion protein after 6 h in inducing conditions (MM-NO_3). Bar = 30 μm.

(E) Distribution of cells showing nuclear GFP accumulation in function of cell length. Quantification is the result of measurement of two independent experiments, counting more than 100 cells each. Mean and SD are shown.

found no hygromycin resistant cells, indicating that *atm1* was an essential gene. We decided to focus on the characterization of Atr1. Sequence comparisons of *U. maydis* Atr1 with orthologs from different organisms revealed the presence of conserved domains, including the PIKK-specific domains FAT and FATC (Bosotti et al., 2000), suggesting that *U. maydis* Atr1 is a bona fide Atr1 ortholog (Figure 2B). Consistently, we found that cells lacking Atr1 were extremely sensitive to several genotoxic insults: UV irradiation, which induces pyrimidine dimers in DNA; hydroxyurea (HU), which inhibits ribonucleotide reductase and therefore affects replication by depletion of deoxynucleotide triphosphates, causing replication fork stalling and collapse; methyl methanesulfonate, which induces DNA alkylation; phleomycin, a radiomimetic drug that causes double-strand breaks in

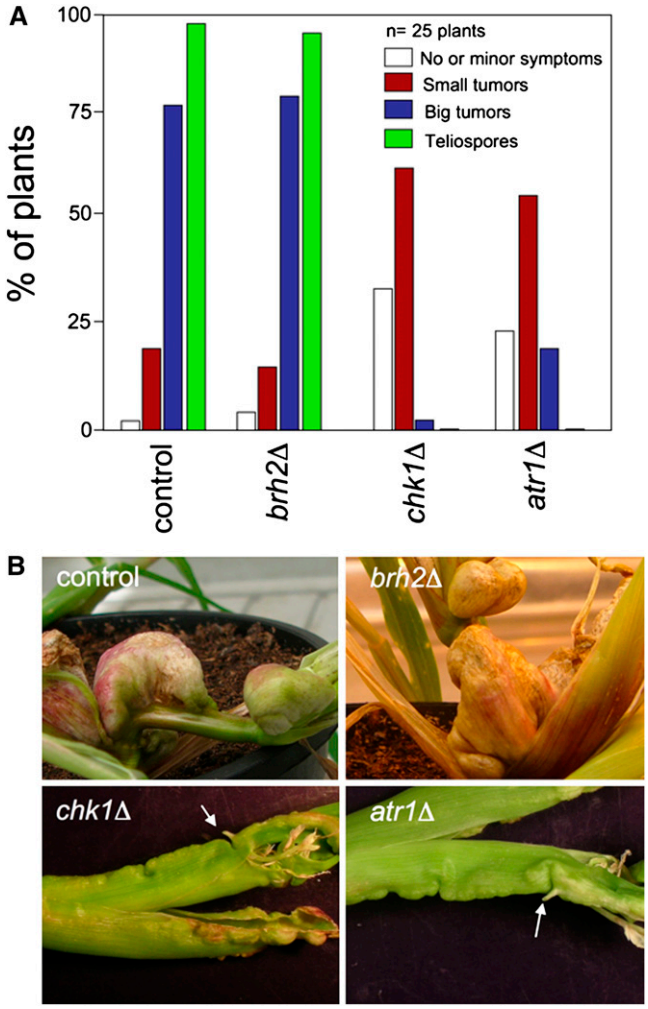

Figure 5. Atr1 and Chk1 Are Required for Full Virulence in *U. maydis*.

(A) Quantification of symptoms in maize plants after 14 d after infection with crosses of wild-type (control, UCM350xUCM520), *brh2*Δ (UCM565-xUCM575), *chk1*Δ (UMP122xUMP129), and *atr1*Δ (UCS9xUCS10) strains.

(B) Morphology of tumors caused by wild-type (control) and compatible combinations of *brh2*Δ, *chk1*Δ, and *atr1*Δ strains. Representative tumors were photographed 16 d after infection. Arrows mark shoot-like structures.

DNA; and ionizing radiation (IR), which generates double-strand breaks (Figure 2C; see Supplemental Figure 2 online). As comparisons we used mutant cells lacking the *chk1* gene as well as cells lacking *brh2*, which encodes a BRCA2-like protein required for homology-directed recombinational repair (Kojic et al., 2002).

Atr1 Phosphorylates and Activates Chk1 in Response to DNA Damage in *U. maydis*

To define relationships between *atr1* and *chk1*, we constructed double mutants of *chk1* and *atr1* and analyzed sensitivity to UV. As control for this epistasis analysis, we also constructed an *atr1 brh2* double mutant strain. Our genetic results suggest that *atr1* acts in the same pathway as *chk1*, since double *chk1 atr1* mutants showed the same sensitivity as *atr1* mutant, while *brh2* seems to work in a distinct pathway than *atr1* since their effects were additive (Figure 3A).

We also analyzed whether Atr1 was required for the accumulation of phosphorylated forms of Chk1 in response to DNA damage agents. Our current view of DNA damage–dependent Chk1 activation in *U. maydis* is that there are two main signals to be detected by DNA surveillance systems: DNA double-strand breaks (induced here by phleomycin treatment) and single-strand DNA tracts as a hallmark of replication stress (caused by HU treatment). We challenged cultures of wild-type and *atr1* mutant strains carrying the Chk1-T7 allele with HU or phleomycin and found that absence of Atr1 abolished the mobility shift in the presence of HU, while in the presence of phleomycin, the shift was only partially abrogated. Distinct behavior in Chk1 phosphorylation with respect to these two DNA damaging agents was previously noted (Pérez-Martin, 2009), suggesting that these two stimuli are transmitted by different signaling pathways, and our results support the idea that at least one of them seems to be totally dependent on Atr1.

Given that phosphorylation seemed to be required for Chk1 activation and that in response to DNA damage Chk1 accumulated in the nucleus, we also analyzed the ability of Chk1 to translocate to the nucleus in response to DNA damage in the absence of Atr1. We examined the subcellular localization of green fluorescent protein (GFP)-tagged Chk1 in the presence of either HU or phleomycin (Figure 3C). While control cells showed a clear nuclear accumulation of the fluorescent signal in the presence of these DNA damaging agents, in the absence of Atr1, GFP-tagged Chk1 failed to accumulate in the nucleus. This response was dramatic in the presence of HU but somewhat less obvious in the presence of phleomycin, mirroring the above phosphorylation results (Figure 3D).

Atr1-Dependent Phosphorylation of Chk1 Is Required for b-Dependent Cell Cycle Arrest

We analyzed whether Atr1 was required for *b*-induced cell cycle arrest. For this, we deleted the *atr1* gene in strains expressing the homeoprotein b-complex heterodimer (AB33 background). We observed that deletion of *atr1* in this genetic background resulted in elongated cells. To distinguish the *b*-induced filaments from such a cell population background, the haploid strains used expressed a GFP fusion to a nuclear localization signal under control of the *dik6* promoter, which is specifically activated by the b-complex heterodimer (Flor-Parra et al., 2006). In this way, only cells expressing the *b*-dependent program produced a fluorescent nuclear signal. We found that in the control strain, almost the totality of the *b*-expressing cell population carried a single nucleus, while *chk1*Δ and *atr1*Δ filaments frequently carried more than two nuclei (Figures 4A and 4B), which was consistent with a defect in the ability to arrest entry into mitosis. These results suggest that most likely Atr1 is the PIKK kinase involved in the activation of Chk1 in response to *b*-induction.

To gain additional support for this idea, we investigated whether Atr1 was required for the accumulation of phosphorylated forms

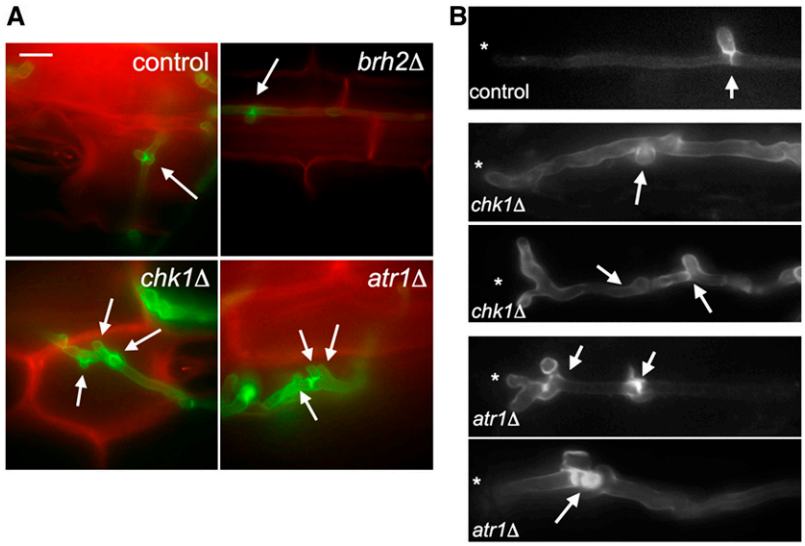

Figure 6. Atr1 and Chk1 Are Required for Normal in Planta Proliferation of Hyphae.

(A) Images of plant tissues 2 d after infection with the indicated crosses of fungal cells. Hyphae (stained by WGA-AF488; green) grow intracellularly in epidermal cells of maize (stained by PI; red). Arrows mark clamp-like connections. Bar = 10 μm.
(B) Examples of aberrant hyphal morphologies found in *chk1*Δ and *atr1*Δ filaments in planta. Asterisk marks the hyphal tip. Arrows denoted aberrant clamp-like structures.

of Chk1 in response to the induction of the *b*-dependent program. Consistently, we found that there was no Chk1 phosphorylation in cells lacking Atr1 after *b*-induction (Figure 4C). In addition, given that phosphorylation seemed to be required for Chk1 activation and nuclear accumulation, the relationships between phosphorylation and nuclear localization in response to the induction of the *b*-dependent program were also investigated. When AB33-derived cells carrying a Chk1-GFP fusion were induced to produce filaments, we observed a clear GFP signal accumulation in the nucleus (Figure 4D). As we already noted, Chk1 activations seems to be a transient response and the nuclear GFP signal disappears in long filaments (Mielnichuk et al., 2009). To quantify this apparently transient response, we measured cells (n = 100 cells, two independent experiments) producing filaments of different length and plotted against the presence or not of a nuclear GFP signal (Figure 4E), confirming that only shorter filaments (i.e., early stages) showed nuclear accumulation of Chk1. When similar measures were performed in AB33-derived cells carrying a Chk1-GFP fusion but lacking the *atr1* gene, we found that no cells showed accumulation of nuclear GFP signal at any stage (Figures 4D and 4E).

Atr1 and Chk1 Have Roles during Pathogenic Growth in Planta

U. maydis infection of maize results in anthocyanin pigment production by the plant and the formation of tumors that are filled with proliferating fungal cells that eventually differentiate into black teliospores (Banuett and Herskowitz, 1996). We tested strains defective either in Chk1 or Atr1 for pathogenicity during the infection process. We found that >25% of the plants showed no or minor symptoms such as chlorosis (Figure 5A). This result can be easily attributed to impaired formation of a functional infective filament, as a consequence of impaired cell cycle arrest during dikaryon establishment (Mielnichuk et al., 2009). However, once plants were infected with these mutant strains, they rarely showed big tumors, and even in these rare occasions, no teliospores were found in these tumors. A striking feature observed in tumors induced by *chk1*Δ and *atr1*Δ strains is the development of small, shootlike structures (Figure 5B). Such structures were described previously in plants infected by *U. maydis* mutants exhibiting overactivation of cAMP cascade and were attributed to a defective fungal development inside the plant (Krüger et al., 2000).

These results strongly suggest roles of Chk1 and Atr1 beyond the initial steps of infection. A possible explanation for these results might be in the compromised ability of the mutant cells to deal with DNA damage occurring during proliferation inside the plant in response to the plant defense system (i.e., reactive oxygen and/or nitrogen species). However, this explanation seems unlikely since the DNA damage–sensitive *brh2* mutant cells complete the life cycle at levels similar to wild-type cells (Figure 5A). This suggests that the Atr1-Chk1 axis has some additional role in the pathogenic process beyond signaling repair of DNA damage.

Chk1 and Atr1 Are Required to Maintain the Dikaryotic Growth

We tested whether proliferation inside the plant was affected by the disruption of Atr1-Chk1. Infected plant tissue was stained with Alexa-Fluor–labeled wheat germ agglutinin (WGA-AF488), a

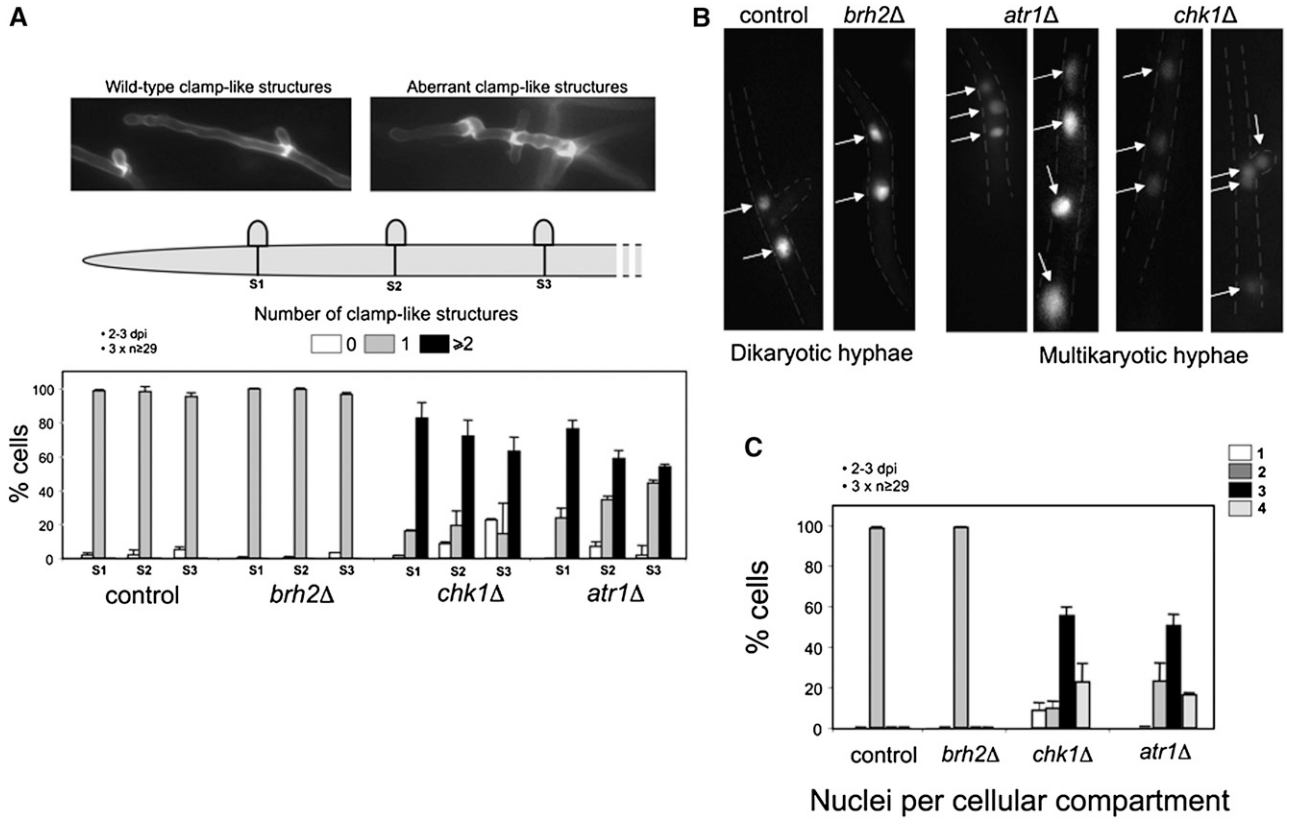

Figure 7. Atr1 and Chk1 Are Required for Dikaryon Maintenance in Planta.

(A) Distribution of clamp-like structures in mutant cells. Infected plant tissue (obtained 2 to 3 d after infection [dpi]) with the indicated strains was stained with WGA-AF488 to detect hyphae and PI to mark maize cells. Then individual hyphae were counted and sorted in relation to the number of clamp-like structures observed at the three more apical septa (S1, S2, and S3). Control (wild type) and *brh2*Δ filaments showed the expected distribution of one clamp-like structure per septum. Filaments defective in *chk1* or *atr1* showed a clear deviation of this pattern. The graph shows the result from three independent experiments, counting more than 29 filaments each. Means and SDs are shown. Top images exemplify typical wild-type (left) or mutant (right) hyphae.

(B) Examples of wild-type and the indicated mutant hyphae expressing a nuclear localized 3xGFP to detect nuclei. Wild-type hyphae contain two nuclei per cell as observed for *brh2*Δ. The *atr1*Δ and *chk1*Δ hyphae contain multiple nuclei per cell compartment. The distribution of the nuclei with respect to the clamp-like structures is given in the lateral illustrations.

(C) Quantification of the number of nuclei per cellular compartment in hyphae from infected plant tissue with the indicated strains. The graph shows the result from three independent experiments, counting more than 29 filaments each. Means and SDs are shown.

lectin that binds to chitin, enabling detection of fungal cell walls, and with propidium iodide, to visualize plant membranes (Doehlemann et al., 2009). *U. maydis* grows as a dikaryon during its pathogenic state. Previous work (Scherer et al., 2006) showed that clamp-like structures were involved in the distribution of nuclei in hyphae and thereby in the ability to proliferate inside the plant. Consistently, we found that in wild-type and *brh2*Δ filaments, single clamp-like structures were placed at the position of septum formation. However, *atr1*Δ and *chk1*Δ mutants were impaired in proliferation, and hyphae showed aberrant formation and variable distribution of clamp-like structures (Figures 6A and 6B). To quantify these defects, individual hyphae were counted and sorted in relation to the number of clamp-like structures observed at the three more apical septa (Figure 7A). Wild-type and *brh2*Δ filaments showed the expected distribution of one clamp-like structure per septum. However, filaments defective in *chk1* or *atr1* showed a clear deviation from this pattern, with apical septa that carry more than one clamp-like structure. Similar defects in clamp-like formation were observed in plants infected with compatible haploid cells carrying the *chk1*$^{T394A\ S448A}$ allele (see Supplemental Figures 1 and 3 online).

Since formation of clamp-like structures is directly related to the process of nuclear division, we wondered whether nuclear distribution was also affected by the disruption of Atr1 or Chk1. To visualize fungal nuclei in infected plants, we used strains carrying a triple GFP gene fused to a nuclear localization sequence under the control of the constitutive promoter P_{tef1} (Flor-Parra et al., 2006). While wild-type and *brh2*Δ filaments carried two nuclei per cellular compartment, *atr1*Δ and *chk1*Δ mutants carried variable combinations from 1 to 4 (Figures 7B and 7C).

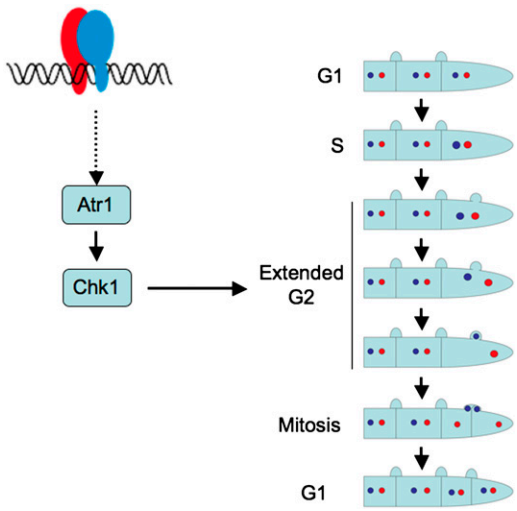

Figure 8. Working Model of the Role That Atr1-Chk1 Play during Pathogenic Growth *of U. maydis* inside the Plant.

During dikaryon cell division, one nucleus enters and divides in the developing clamp cell, whereas the other divides in the main hypha, with the result that mitosis occurs in two distinct cell compartments. Most likely these processes take place during G2 phase. Since such a nuclear ballet will need an extended G2 period, we propose that the role of Atr1 and Chk1 during dikaryotic cell cycle is to synchronize nuclei as well as provide an extended window during G2 phase to allow the above-mentioned process.

These observations suggest that Chk1 and Atr1 have roles in appropriate nuclear segregation and therefore that in their absence, dikaryotic cells replicate aberrantly, most likely causing the observed proliferation defects in planta.

DISCUSSION

Dikaryon cell division involves nuclear migration and sorting of the divided nuclei to ensure that each daughter cell inherits a balance of each parental genome (Casselton, 2002). This cell cycle relies on a synchronized nuclear division and the development of clamp connections, a specialized projection formed close to the position of the future septum formation. One nucleus enters and divides in the developing clamp cell, whereas the other divides in the main hypha, with the result that mitosis occurs in two distinct cell compartments. It is not known in which cell cycle stage these processes take place, but G2 phase is most likely, as it happens after nuclear DNA duplication and before mitosis. Moreover, the movement of one of the nuclei into the clamp cell is reminiscent of the movement of the diploid nucleus into the daughter bud during vegetative cell division in *U. maydis*, a process occurring at the G2/M transition (Straube et al., 2005). Such a nuclear ballet most likely will need an extended G2 period to proceed. One of the roles of the Atr1-Chk1 axis during DNA damage response is to provide an elongated G2 phase to cells to allow time to repair DNA and resolve other problems before mitosis starts (Toettcher et al., 2009). On this basis, we propose that the role of the Atr1-Chk1 axis during the dikaryotic cell cycle is to synchronize nuclei as well as to provide an extended window during G2 phase to enable the above-mentioned process (Figure 8). It could be well that in the absence of the Atr1-Chk1 axis, the nuclear cycle, clamp cell formation, and cytokinesis occur asynchronously resulting in the defects such as observed in mutant hyphae inside the plant.

Since b proteins are required for synchronization between cell cycle and cytokinesis (Wahl et al., 2010), we propose that in normal conditions it is the timely activation of the Atr1-Chk1 axis by the b homeodomain protein that produces accurate control of cell cycle. We have no clue about how the b protein activates this cascade. Our preliminary attempts to correlate expression of compatible *b* alleles with an increase in *chk1* or *atr1* transcription were negative (data not shown). Chk1 activation is linked to DNA damage in vegetative cells, and our results indicated that Atr1 is also required for the response to DNA damage. Therefore, it is tempting to speculate that *b*-induction could be associated with some class of DNA damage that triggers this kinase cascade during b-dependent filament formation. It is worth noting that b proteins activate the transcription of a gene encoding a putative DNA polymerase β (Brachmann et al., 2001), which belongs to the family X of DNA polymerases that are described to be involved in a number of DNA repair processes (Ramadan et al., 2004). On the other hand, several lines of evidence suggest that it is unlikely that activation of the Atr1-Chk1 cascade during *b*-induction is related to DNA damage. First, in previous studies using the formation of Rad51 foci as reporter for active DNA repair, there was no evidence for DNA damage associated with the induction of the infective hyphae (Mielnichuk et al., 2009). Second, we observed no defect in the ability to arrest the cell cycle (see Supplemental Figure 4 online) or to infect plants (Figure 5A) in cells lacking Brh2, which is required for DNA repair by homologous recombination (Kojic et al., 2002). Finally, preliminary research indicated that cells lacking the PolX polymerase were able to arrest cell cycle at levels comparable to wild-type cells (see Supplemental Figure 4 online). Two recent reports showed that activation of the DNA damage response cascade can be triggered in the absence of DNA damage by stable association of elements of the cascade with chromatin (Bonilla et al., 2008; Soutoglou and Misteli, 2008). Whether a similar mechanism could explain our observations in *U. maydis* will require additional research.

There are increasing numbers of reports indicating that the ability of the DNA damage response cascade to modulate cell cycle progression can be used during developmental processes. Some of these processes were linked with limited DNA damage as happens in B cell differentiation (Sherman et al., 2010) or even in the absence of apparent DNA damage, such as in the midblastula transition in *Drosophila melanogaster* embryos (Sibon et al., 1997) or in the asynchronous division at two-cell-stage *Caenorhabditis elegans* embryos (Brauchle et al., 2003). The surprising finding that a regulatory cascade involved in DNA damage responses plays a role in a fungal developmental process mirrors these previous results and reinforces the emerging idea that checkpoint cascades may have roles beyond cell surveillance by virtue of their ability to interact with cell cycle machinery.

Table 1. Strains Used in This Work

Strain	Relevant Genotype	Reference
UCM350	*a1b1*	Kojic et al. (2002)
UCM520	*a2b2*	Kojic et al. (2002)
UMP122	*a1b1 chk1*Δ	Pérez-Martín (2009)
UMP129	*a2b2 chk1*Δ	Mielnichuk et al. (2009)
UCS9	*a1b1 atr1*Δ	This work
UCS10	*a2b2 atr1*Δ	This work
UCM565	*a1b1 brh2*Δ	Kojic et al. (2002)
UCM575	*a2b2 brh2*Δ	Kojic et al. (2002)
UCS22	*a1b1 atr1*Δ *chk1*Δ	This work
UCS27	*a1b1 atr1*Δ *brh2*Δ	This work
AB33	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1*	Brachmann et al. (2001)
UCS31	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1 chk1-T7*	This work
UMP114	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1 chk1*Δ	Mielnichuk et al. (2009)
UCS32	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1 chk1-T7 atr1*Δ	This work
UMP183	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1 chk1-T7*$^{T394A\ S448A}$	This work
UMP190	*a1b1 chk1-T7*$^{T394A\ S448A}$	This work
UMP191	*a2b2 chk1-T7*$^{T394A\ S448A}$	This work
UMP162	*a1b1 chk1-T7*	This work
UMP207	*a1b1 atr1*Δ *chk1-T7*	This work
UMP111	*a1b1 chk1-3GFP*	Pérez-Martín (2009)
UCS15	*a1b1 atr1*Δ *chk1-3GFP*	This work
UCS20	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1* P_{dik6}*:NLS-3GFP*	This work
UMP112	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1* P_{dik6}*:NLS-3GFP chk*1Δ	Mielnichuk et al. (2009)
UCS21	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1* P_{dik6}*:NLS-3GFP atr1*Δ	This work
UMP196	*a1 b1* P_{tef1}*:NLS-3GFP*	This work
UMP197	*a1 b1* P_{tef1}*:NLS-3GFP brh2*Δ	This work
UMP198	*a1 b1* P_{tef1}*:NLS-3GFP atr1*Δ	This work
UMP199	*a1 b1* P_{tef1}*:NLS-3GFP chk1*Δ	This work
UMP133	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1 chk1-3GFP*	Mielnichuk et al. (2009)
UMP208	*a2* P_{nar1}*:bW2* P_{nar1}*:bE1 chk1-3GFP atr1*Δ	This work

METHODS

Ustilago maydis Genetic Methods

U. maydis strains are listed in Table 1. Manipulations with *U. maydis*, culture methods, gene disruption and gene transfer procedures, survival after DNA damage, and plant infections have been described previously (Castillo-Lluva and Pérez-Martín, 2005; Flor-Parra et al., 2007; Mielnichuk and Pérez-Martín, 2008; Mielnichuk et al., 2009). Protein extracts, immunoprecipitations, and immunoblot analysis were performed as described previously (García-Muse et al., 2004; Sgarlata and Pérez-Martín, 2005). To detect the phosphorylated forms of Chk1, T7-tagged Chk1 proteins were immunoprecipitated using an anti-T7 antibody (Sigma-Aldrich) from cell extracts and subjected to SDS-PAGE in 8% acrylamide/0.1% bisacrylamide, pH 9.2, gels. Blots were incubated with anti-T7-horseradish peroxidase (Sigma-Aldrich) and visualized using enhanced chemiluminescence (Renaissance; Perkin-Elmer).

Null mutants were constructed by replacing the entire open reading frames with cassettes expressing resistance to antibiotics by standard methodology (Brachmann et al., 2004). Briefly, a pair of DNA fragments flanking the *atr1* open reading frame were amplified and ligated to a gene cassette encoding hygromycin resistance and flanked by *Sfi*I sites. The 5′ fragment spans from nucleotide −1967 to nucleotide −29 (considering the adenine in the ATG as nucleotide +1), and it was produced by PCR amplification using the primers ATR1-22 (5′-TTAATTAAGCAGATCCACTGCTGAACGGGTTTC-3′) and ATR1-3 (5′-GGTGGCCATCTAGGCCTTCCTTAGGCTTGGACACTGGAGATCAGT-3′). The flanking 3′ fragment was obtained after PCR amplification with primers ATR1-4 (5′-ATAGGCCTGAGTGGCCACGGTTTGCAGCTGCATACAGTAGGATAT-3′) and ATR1-23 (5′-TTAATTAAGGAACTCATCAGCGTGTGGAACCGA-3′) and spans from nucleotide +7912 to nucleotide +9962.

Microscopy

To visualize fungal hyphae in plant by wheat germ agglutinin-Alexa fluor 488 (WGA-AF488; Invitrogen) and propidium iodide (PI) (Sigma-Aldrich), samples 2 to 3 d after infection of maize (*Zea mays*) leaves were incubated in staining solution (1 μg/mL PI, 10 μg/mL WGA-AF488, and 0.02% Tween 20) for 30 min and washed in 1× PBS, pH 7.4 (Doehlemann et al., 2009). Analysis of the infection stages was done using a Deltavision wide-field microscope (Applied Precision). Image deconvolution was performed using z-series of between 10 and 15 focal planes, acquired at 0.5-μm intervals. Image processing was performed using Adobe Photoshop CS2 and Canvas 8.0 (Deneba).

To quantify the clamp-like structures in fungal hyphae, plant leaves 2 to 3 d after infections were analyzed. Individual hyphae where the hyphal tip as well as the penetration points could be observed were analyzed, quantifying the number of clamp-like structures. To quantify the number of nuclei per cellular compartment, maize leaves 2 to 3 d after infections were analyzed. Only nuclei in individual hyphae were quantified.

Sequence Analysis

Alignments were made with ClustalW (Thompson et al., 1997). Phylogenetic dendrograms were constructed using MEGA 2.1 (Kumar et al., 2001)

with the minimum evolution or maximum parsimony algorithm and gap deletion option.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers JF690671 (Atm1) and JF690672 (Atr1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phosphorylation of Chk1 Is Required for Normal in Planta Proliferation of Hyphae.

Supplemental Figure 2. Complementation of *chk1* and *atr1* Deletion Mutants.

Supplemental Figure 3. Single Phosphorylation Mutants in Chk1 Showed Wild-Type Phenotypes with Respect to Cell Cycle Arrest and Infection.

Supplemental Figure 4. Absence of Brh2 or PolX Does Not Affect the Ability of the b-Complex to Arrest Cell Cycle.

Supplemental Data Set 1. Sequence Alignment of Atr- and Atm-Like PI3K Kinases.

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Dikaryotic cell cycle in the phytopathogenic fungus *Ustilago maydis* is controlled by the DNA damage response cascade

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In a large group of fungi, mating results in a dikaryon, a cell in which the two nuclei—one from each parent cell—share a single cytoplasm for a period of time without undergoing nuclear fusion. The dikaryon stage is typical in the life cycles of many fungal species primarily in the Basidiomycota, a large group that includes mushrooms, bracket fungi and many phytopathogenic fungi, such as the corn pathogen *Ustilago maydis*. Recently, we described that in *U. maydis* two conserved DNA-damage checkpoint kinases, Chk1 and Atr1, work together to control the dikaryon formation. However, how this pathway is activated during the dikaryon formation and how its activation/deactivation is coordinated with the different cell cycle phases is unknown. Here we propose and discuss several hypothesis to address these questions.

Key words: DNA damage response, phytopathogenic fungus, cell cycle, corn smut, *Ustilago maydis*, virulence

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In the phytopathogenic fungus *Ustilago maydis*, virulence and sexual development are intricately interconnected.[1] A prerequisite for generating the infectious stage is the mating of two compatible budding haploid cells to generate, after cell fusion, an infective dikaryotic filament. Once the fungus enters the plant tissue, the dikaryotic state dominates the period of growth occurring during the infectious phase. Dikaryons are cells in which two nuclei, one from each parent cell, share a single cytoplasm for a period of time without undergoing nuclear fusion.[2] Maintenance of the dikaryotic state requires an elaborated cell cycle that relies on a synchronized nuclear division and the development of specialized projections (known as clamp connections) formed close to the position of the future septum formation. One nucleus enters and divides in the developing clamp cell, whereas the other divides in the main hypha, with the result that mitosis occurs in two distinct cell compartments.[3] These processes take place during G_2 phase, which has to be properly enlarged for this purpose.

The establishment and maintenance of dikaryotic growth is controlled by a heterodimeric homeodomain transcription factor, the b-complex, which subunits (bW and bE) are provided by each compatible mating partner.[4] For a long time, a connection between the b heterodimer and the cell cycle control was predicted, although the details behind these connections were largely unknown.[5] Recently, we described that Chk1 and Atr1, two DNA-damage checkpoint kinases, were activated in response to the formation of b-heterodimer and that this activation resulted in a transient G_2 cell cycle arrest, most likely providing the time window required for appropriated dikaryon cell division.[6,7] The absence of either Chk1 or Atr1 kinases resulted in defects in the ability of the dikaryotic cells to divide properly and therefore proliferation was affected.

Chk1 and Atr1 kinases are part of a signaling cascade devoted to cope with DNA damage, which role is conserved in a large number of eukaryotic organisms including *U. maydis*.[8] The described new role of Atr1 and Chk1 during pathogenic development in *U. maydis* fits in the emerging view that elements from the DNA damage response cascade can be utilized to modulate developmental processes in virtue to their ability to interact with cell cycle

machinery elements.[9] In *U. maydis* there are two major questions concerning these connections that remain to be uncovered, and our current view and ideas about these questions are discussed below.

How a DNA Damage Response Pathway is Activated by a Transcriptional Factor during Dikaryon Formation?

A main question to be answered concerns how the b heterodimer, a transcriptional factor, activates the Atr1-Chk1 cascade, which in normal conditions responds to DNA damage. Attempts to correlate activation of Atr1-Chk1 cascade during *b*-induction with massive DNA damage—using the formation of Rad51 foci as reporter for active DNA repair—were unsuccessful.[7] One possibility could be that the putative DNA damage is different from double strand break damage, so alternative DNA repair pathways such as base excision repair (BER) are recruited, and therefore no need for Rad51. In other eukaryotic systems, BER-mediated signaling is independent on Atr1-Chk1, but perhaps in *U. maydis* is more simplified than in higher eukaryotes for instance, and involves Atr1-Chk1 pathway.[10] Another possibility is that a limited DNA damage (for instance, a single double strand break, not detectable using the Rad51-GFP reporter) induced by gene products regulated by b heterodimer, was responsible of the developmental activation of the DNA damage cascade during the induction of the virulence program in *U. maydis*. This explanation was inspired in the role of HO endonuclease during mating-type switching in *Saccharomyces cerevisiae*.[11] In opposition to these explanations suggesting coupling between DNA damage and *b* induction is worth to say that no defect in the ability to arrest cell cycle or to infect plants was apparent in cells lacking Brh2, a BRCA2-like protein that is required for DNA repair.[12] An alternative to DNA damage is that *b* induction may alter the kinetics of progression through S phase (perhaps via depletion of nucleotide pools or delaying the firing of late origins). In fact, genes involved in DNA replication such as those encoding putative subunits of polymerase (*pol2*, um01008; *pol1*, um 04529), DNA replication licensing factor (um06402) or ribonucleoside reductase (*rrn1*, um11750) are downregulated after b-induction.[13] However, it has not been addressed whether such a gene transcription downregulation is the cause of cell cycle arrest or whether the transcription of these genes is downregulated because the cell cycle arrest (in other words, is not clear whether they are cause or consequence). One relatively simple way to test this idea, the use of FACS analysis to determine whether *b* induction prolongs S phase, indicated no obvious defect in S-phase progression in cells producing the b heterodimer.[7] One appealing hypothesis is that a few replication origins could be more sensitive and that these origins could be responsible of the cell cycle arrest. Currently we are characterizing a wide collection of replication origins in *U. maydis* and carrying out bi-dimensional analysis of replication intermediates trying to detect differences. Finally a third alternative to explain how a transcription factor triggers a DNA-damage cascade is based in two recent reports showed that activation of DNA damage response cascade can be triggered in the absence of DNA damage by stable association of elements of the cascade with chromatin.[14,15] Whether a similar mechanism could explain our observations in *U. maydis* will need additional research.

How to Alternate Activation/Deactivation Cycles of Atr1-Chk1 Cascade Coupled to Cell Cycle Transitions in the Dikaryon?

The second main question to be addressed is how the cell cycle arrest is intermittently released during biotrophic development. The current idea is that Atr1-Chk1 cascade has to be activated by the b heterodimer every cell cycle, to provide the extended G_2 phase, but that once nuclei are separated in different cell compartments (one in the clamp cell, the other in the main hypha) this signal cascade has to be shut off, to allow the G_2/M transition. The predicted downregulation must occur at least at two different levels: the transcriptional activity of b heterodimer has to be inhibited and the Chk1-Atr1 signaling has to be stopped. Downregulation of b heterodimer occurs most likely through the activity of the Clp1 (Clampless1) protein. This factor was first described in the mushroom *Coprinopsis cinerea* as needed for clamp formation as well as for the distribution of nuclei during cell division of the dikaryon, via its ability to interact with the A-complex (the ortholog of b heterodimer in this mushroom).[16] In *U. maydis*, Clp1 interaction with bW blocks b-dependent functions, such as the b-dependent G_2 cell cycle arrest: for instance, the forced expression of *clp1* in strains where filamentation is induced by an active bE/bW heterodimer suppresses the cell cycle block. Moreover, *clp1* mutants are unable to release the bE/bW-triggered cell cycle arrest.[17] Interestingly *clp1* transcription is indirectly activated by the b heterodimer, via the induction of the transcriptional activator Rbf1, so the downregulation of b heterodimer is the result of a negative feedback by end product.

How the second level of downregulation, the attenuation of Atr1-Chk1 signal, occurs is unknown. Recently it has been described that in mammalian cells the Akt/PKB kinase is able to override DNA damage-induced G_2 arrest, via phosphorylation of Chk1.[18] This way, the cell becomes refractory to Atr1-Chk1 activation late in G_2 before the onset of mitosis. Interestingly, in *U. maydis*, the transcriptional levels of *ukb1*, encoding the putative ortholog of PKB, are increased during *b*-induction, via Rbf1, in a similar way as *clp1* is upregulated.[13] Moreover, *U. maydis* cells defective in *ukb1* are affected in proliferation in planta, producing plant symptoms that remind those obtained when plants were infected with *chk1* or *atr1* mutants (small tumors and absence of teliospore production).[19] We entertained the hypothesis that as it happens in mammalian cells, in *U. maydis* Ukb1 could downregulate the Atr1/Chk1 pathway through direct phosphorylation of Chk1.

On basis of these ideas, our hypothesis proposes that attenuation of G_2 arrest occurs at two levels: an immediate shut off of the signaling through the Atr1/Chk1 pathway mediated by the inhibition of Chk1 by the PKB-like kinase Ukb1; and a posterior second level of transcriptional shut-off of b heterodimer by the

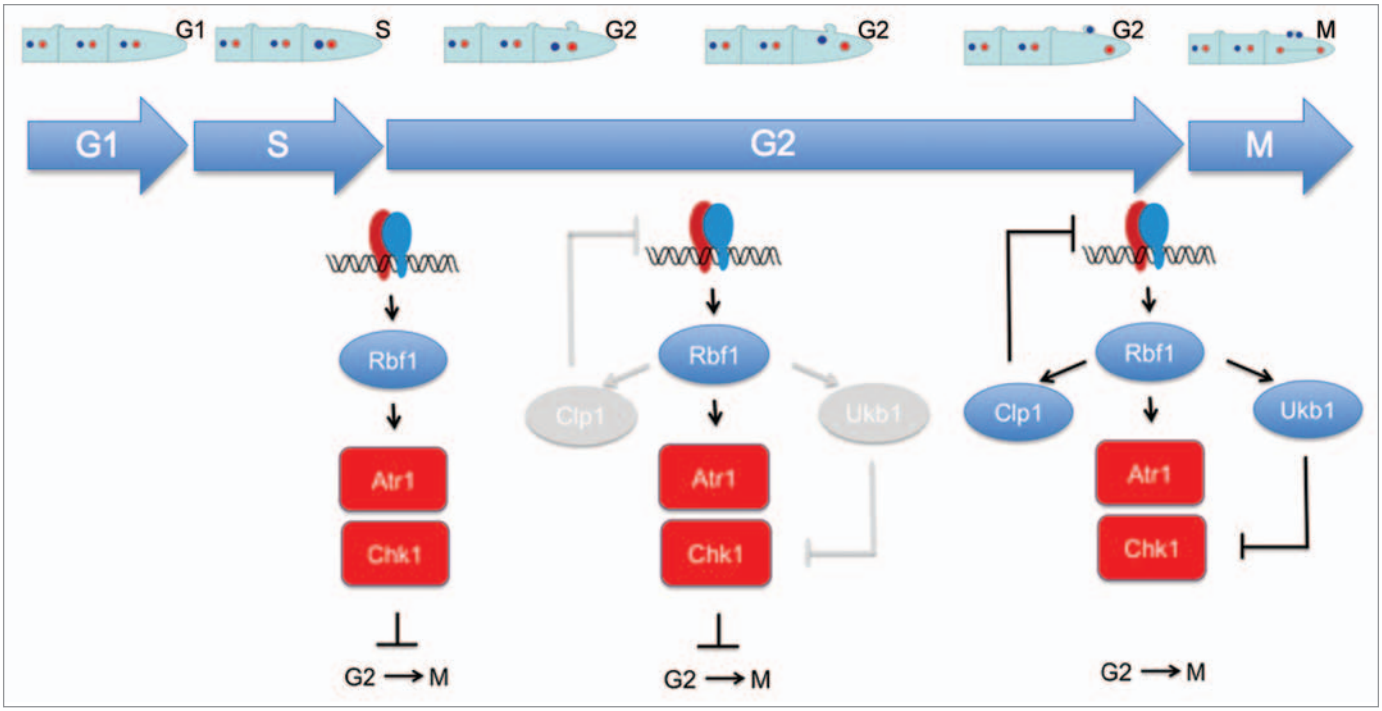

Figure 1. Hypothetical model of b-dependent G_2 enlargement during dikaryon cell cycle. The b heterodimer activates a transcriptional cascade, which includes the Rbf1 regulator, resulting in the activation of the Atr1-Chk1 cascade. Chk1 inhibits the Cdc25 phosphatase resulting in a delay in G_2/M transition, providing the expected enlargement of G_2 phase required for the formation of clamp-like structures as well as the associated nuclear movements. During this enlarged G_2 phase, the transcriptional activity of Rbf1 also generates the accumulation of two regulators: the Clp1 protein, which inhibits the b heterodimer; and the Ukb1 kinase, that is proposed to inhibit Chk1. Once these two regulators reach a threshold, the pathway is downregulated at transcriptional level (by Clp1-mediated inhibition of b heterodimer) as well as at the level of the signal cascade (by the Ukb1-mediated inhibition of Chk1), enabling the transition from G_2 to M phase.

Clp1 protein (**Fig. 1**). Both negative regulators, Ukb1 and Clp1 are end-products of a transcriptional cascade formed by b heterodimer and its downstream regulator Rbf1. Clp1 inhibits b heterodimer, so we propose that a transcription network defined by sequential waves of expression of transcription factors might function independently of any extrinsic control. This transcriptional network may oscillate independently of the Cdk-cyclin oscillator (responsible of cell cycle transitions) although they are coupled in a manner that it provides the proper timing to ensure the cell cycle events. A transcriptional network model in which transcription factors expressed in one step bind to the promoters of genes encoding transcription factors that function in a subsequent step and inhibits the previous one were recently proposed as an emergent property of the transcription factor network that functions as a cell cycle oscillator independently of, and in tandem with, the CDK oscillator, during cell cycle transitions.[20] From our hypothesis a few predictions could be made, and one of these is that altering the transcriptional rate of the promoters responsible of intermediate regulatory factors (e.g., *clp1* or *ukb1*) might affect the length of G_2 phase and thereby the ability to properly form the dikaryon. These predictions are being tested currently in our laboratory.

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